**Patrícia Marques Rodrigues**

Licenciatura em Bioquímica

# Nevirapine in an animal model of pre-diabetes: study of drug pharmacokinetic and its effects on fasting glycemia and insulin resistance

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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# Abstract

The increased incidence of type II diabetes has emerged as a major concern in controlled human immunodeficiency virus (HIV) infection. There is a general lack of data to support the best combined antiretroviral therapy (cART) option to treat HIV-patients with pre-diabetes and nevirapine has been described has a glucose-friendly antiretroviral. On the other hand, it is known that diabetes could influence the pharmacokinetics of several drugs. This aspect is particularly relevant for drugs with narrow therapeutic window, which is the case of nevirapine.

To understand if nevirapine is a good choice for pre-diabetic HIV-patients, the effect of insulin resistance in NVP pharmacokinetics as well as the effect of nevirapine on insulin resistance, fasting glycemia and mean arterial pressure was evaluated. Moreover, nevirapine effect on thiols content, an endogenous antioxidant defence system, was also evaluated. To achieve the main goal four groups of female *Wistar* rat were used: a control group, a control group treated with nevirapine, an insulin resistant group and an insulin resistant group treated with nevirapine.

An influence of a pre-diabetic status on nevirapine pharmacokinetic was found. Nevirapine and its phase I metabolites presented changes in disposition and the metabolite profile pattern was changed. Moreover, nevirapine, in a pre-diabetic perspective, is associated with a beneficial effect on fasting glycemia, while it has no effect on sensitivity to insulin or in arterial pressure. Furthermore, nevirapine is associated with a lower degradation of total glutathione.

Nevirapine might be a good option for HIV-infected patients at higher risk of develop diabetes or in pre-diabetic condition. Moreover, while further studies are necessary to consolidate this issue, nevirapine might be less toxic in pre-diabetes. Although, the decreased bioavailability of nevirapine in pre-diabetes requires special attention, as an adjustment of nevirapine dose might be required in this subpopulation.

**Keywords: nevirapine, pre-diabetes, insulin resistance, drug toxicity, antioxidant defense system**

# Resumo

A incidência de diabetes tipo II na infecção pelo vírus da imunodeficiência humana (VIH) tem vindo a aumentar. A evidência científica que justifique a melhor opção terapêutica para doentes VIH positivos pré-diabéticos é actualmente muito limitada. No entanto, a nevirapina tem-se destacado por apresentar uma influência favorável no metabolismo da glucose. Por outro lado, as alterações na farmacocinética dos medicamentos num contexto de diabetes estão bem documentadas. Este aspecto é de grande importância para um fármaco com uma janela terapêutica estreita, de que é exemplo a nevirapina.

De forma a explorar a indicação da NVP para indivíduos pré-diabéticos VIH positivos, o efeito da insulinorresistência na farmacocinética da nevirapina, assim como o efeito da nevirapina na resistência à insulina, glucose em jejum e pressão arterial foi avaliado. Para atingir este objectivo, recorreu-se a quatro grupos constituídos por ratos *Wistar* fêmeas: grupo controlo, grupo controlo tratado com nevirapina, grupo insulinorresistente e grupo insulinorresistente tratado com nevirapina.

A influência da pré-diabetes na farmacocinética da nevirapine foi comprovada manifestando-se como uma variação na disposição da nevirapina e seus metabolitos de fase I, assim como um diferente perfil de metabolitos. Para além disso, o tratamento com nevirapina parece estar associado a uma melhoria dos níveis de glucose em jejum e a uma menor degradação do glutatião. Estes novos dados sugerem que a nevirapina possa ser eleita como uma boa opção para doentes VIH positivos com elevado risco para desenvolver diabetes ou pré-diabetes. Por outro lado, e apesar da necessidade de mais estudos para consolidar esta hipótese, a nevirapine poderá ser menos tóxica num contexto de pré-diabetes. Por último é relevante salientar que a diminuição da biodisponibilidade da nevirapina na pré-diabetes requer uma especial atenção, pela possível necessidade de um ajuste de dose nesta sub-população.

**Palavras-chave: nevirapine, pré-diabetes, resistência à insulina, toxicidade medicamentosa, sistema de defesa antioxidante.**

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# Abbreviations

| | |
|---|---|
| 2-OH NVP | 2-hydroxy nevirapine |
| 3-OH NVP | 3-hydroxy nevirapine |
| 8-OH NVP | 8-hydroxy nevirapine |
| 12-OH NVP | 12-hydroxy nevirapine |
| cART | Combined antiretroviral therapy |
| CYP P450 | Cytochrome P450 enzymatic system |
| Cys | Cysteine |
| Cys-Gly | Cysteinyl-Glycine |
| HAART | Highly active antiretroviral therapy |
| HCys | Homocysteine |
| HIV | Human Immunodeficiency virus |
| HPLC | High-performance liquid chromatography |
| HSu | High-sucrose |
| GSH | Glutathione |
| IR | Insulin resistance |
| ITT | Insulin Tolerance Test |
| $K_{ITT}$ | Constant rate for glucose disappearance in the insulin tolerance test |
| MAP | Mean arterial pressure |
| MC | Methylcellulose |
| NAC | N-acetyl cysteine |
| NNRTI | Non-nucleoside reverse transcriptase inhibitors |
| NRTI | Nucleoside reverse transcriptase inhibitors |
| NVP | Nevirapine |
| PBS | Phosphate buffered saline |
| PD | Pharmacodynamic |
| PI | Protease Inhibitor |
| PK | Pharmacokinetic |
| TIIDM | Type II diabetes mellitus |

# 1. Introduction

## 1.1 Human immunodeficiency virus infection and the combined antiretroviral therapy

The numbers of HIV-infection are tremendous: from 2001 to 2011 this figure increased by 5 million people, reaching 34 million of infected individuals, which correspond to 0.8% of adult population worldwide. In this scary scenario, the health promotion regarding this infection and the availability of combined antiretroviral therapy (cART) in middle 90's have totally revolutionized the prognostic of this infection, which nowadays is considered a chronic condition in properly medicated patients. The good numbers reflect the increased decline in HIV-associated deaths of approximately 600 thousands from 2005 to 2011. Moreover, during the year 2011, the number of persons which had benefit from anti-HIV treatment, increased by 1.6 million people (World Health Organization, 2013). Currently there are more than 20 antiretroviral drugs available, distributed into five major classes which are classified according their mechanisms of action (**Figure 1.1**).

The choice of cART for a particular patient and the optimal times for its start should be in agreement with several elements, such as clinical manifestations, the number of lymphocytes CD4 (<350 cell/$mm^3$), the value of plasma viral load and the presence of co-morbidities. The first-line cART is normally composed by two nucleoside reverse transcriptase inhibitors (NRTI) combined with a third drug which should be preferentially a non-nucleoside reverse transcriptase inhibitor (NNRTI), namely efavirenz or nevirapine (Guerreiro *et al.*, 2012).

As, so far, the cure for HIV-infection has not been found, once a patient starts cART it must persist through his/her life. Moreover, despite the undoubtedly benefit of cART in patient's life expectancy and quality of life, antiretrovirals are associated with several undesirable effects. Moreover, the persistent immune dysfunction and chronic inflammation characteristic of this infection, patient's lifestyle risk factors (smoking, alcohol misuse), polymedication and higher risk for drug–drug interactions potentiate the increased risk of antiretroviral induced toxicity. Additionally, as patients live longer the incidence of aging-associated complications has been increasing in this population (Deeks and Phillips, 2009). In the top of the list are metabolic disorders, including insulin resistance (IR), diabetes mellitus, dyslipidemia and lypodystrophia (Samaras, 2012; Kalra and Agrawal, 2013).

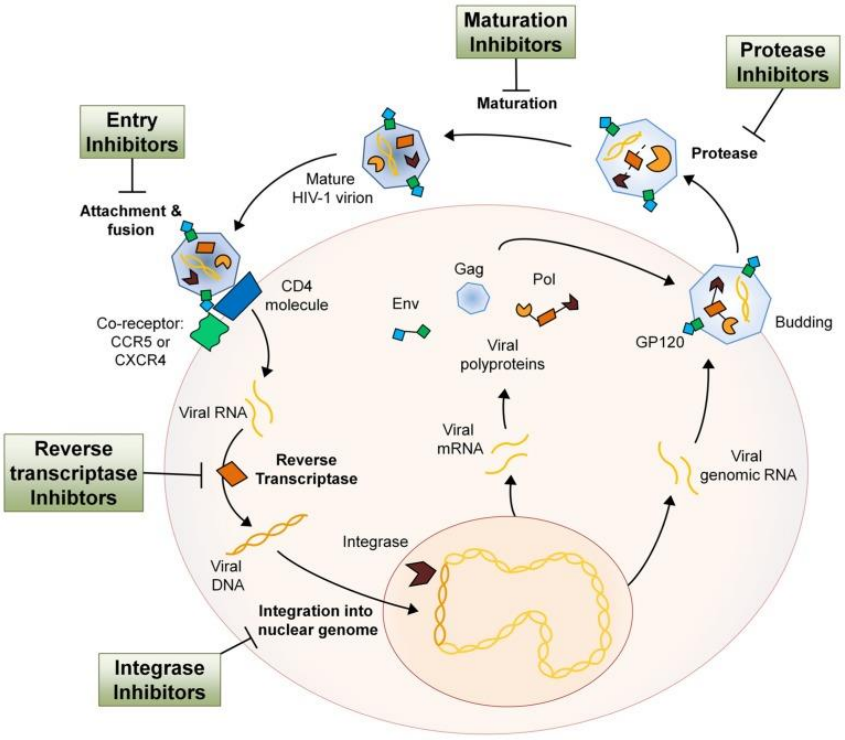


**Figure 1.1 - The life cycle of HIV infection and antiretroviral drug class target. In: (Smith *et al.*, 2013)**
These classes target the following steps in the HIV life cycle: binging and fusion, reverse transcription, integration, transcription, assembly and finally budding. Entry inhibitors interfere with viral entry into the host cell; nucleoside reverse transcriptase inhibitors (NRTI) inhibit the HIV protein reverse transcriptase; non-nucleoside reverse transcriptase inhibitors (NNRTI) also inhibits the reverse transcriptase enzyme but in a non-competitive manner; protease inhibitors (PI) inhibit the protease enzyme and thus the HIV replication; integrase inhibitors (INI) interfere with viral integrase which is responsible for viral DNA interaction in human immune cells; maturation inhibitors disrupt the final step in the processing of the HIV gag protein (De Clercq, 2007; Richards and McCallister, 2008).

## 1.2 Human immunodeficiency virus infection and the combined antiretroviral therapy and type II diabetes

Improved methods for detection of HIV, an earlier HIV diagnosis as well as a better management of cART have translated this infection into lesser morbidity and mortality and a consequent longer lifespan. Although, in clinical practice, it has meant an increase in the chronic complications associated with this infection (Deeks and Phillips, 2009).

As treatment of HIV management develops and the access to therapy improves, the incidence of HIV-associated diabetes is bound to grow (Paik and Kotler, 2011). The range of potential adverse consequences of cART is wide and includes gastro-intestinal disturbance, hepatotoxicity, pancreatitis and mitochondrial toxicity (Kalra *et al.*, 2011). Thus, risk associations between HIV, its treatment, and diabetes have been reported in adults and children (Brown *et al.*, 2005; Samaras *et al.*, 2007). For

instance, in 130 children aged 10 years, it was observed a 13.2% prevalence of IR associated to cART (Beregszaszi *et al.*, 2005). Also, in a young adult cohort of 755 HIV-infected patients with a mean age among 36-48 years, a prevalence rate of 4.5%, 9.4% and 11.9% of diabetes, impaired fasting glucose and hyperinsulinemia was respectively found (Calza *et al.*, 2011).

The mechanism for this is unknown but it is thought to either be due to the infectious/inflammatory process of HIV itself or a form of drug induced toxicity or perhaps through indirect effects. Beyond the conventional risk factors as family history, hypertension, male gender, certain ethnic backgrounds or culture aging (Kilby and Tabereaux, 1998), the individuals infected with HIV have additional factors that contribute to a higher risk. These factors include chronic inflammation caused by the virus, viral factors (viral burden, lower CD4 count and duration of viral infection) (Kilby and Tabereaux, 1998; Capeau *et al.*, 2012), co-pathologies diseases (e.g., chronic hepatitis C infection (HCV)) (Mehta *et al.*, 2003) and factors related with antiretroviral drugs (Lewis *et al.*, 2003; Limone *et al.*, 2003). Despite there is no predominant metabolic effect for each antiretroviral class, the most undesirable metabolic effects have been described for protease inhibitors (PIs) (Murata *et al.*, 2000; Noor *et al.*, 2004; Stanley *et al.*, 2009). The influence of nucleotide reverse transcriptase inhibitors (NRTIs) is controversial and dependent on each particular drug (Mulligan *et al.*, 2000; Justman *et al.*, 2003; Brown *et al.*, 2005; Tien *et al.*, 2007; De Wit *et al.*, 2008). The NNRTI NVP has been described has a drug with a favorable glucose-insulin profile (Eastone and Decker, 1997; De Wit *et al.*, 2008) and was recently indicated has a protective factor for pre-diabetic HIV-infected patients (Srivanich *et al.*, 2010).

The NNRTI-based cART has been usually prescribed as the first-line treatment in resource-limited settings for several years (Sungkanuparph *et al.*, 2006; Katabira and Oelrichs, 2007; Kiertiburanakul *et al.*, 2007; Manosuthi *et al.*, 2007). Moreover, the portuguese guidelines for HIV treatment have recently recommend first-generation NNRTI-containning cART as the preferred option for first-line therapy (Guerreiro *et al.*, 2012).

On the other hand, all NNRTI have potential to hepatotoxic reactions (Pereira *et al.*, 2012a) and strong evidence suggest that it is upon their biotransformation (Pereira *et al.*, 2012b). Moreover, compiling evidence indicates that diabetes might change drug pharmacokinetic (PK) and pharmacodinamic (PD) properties (Dostalek *et al.*, 2012). Importantly, PK variations are particular concerning for drugs with a narrow therapeutic window, which is the case of antiretrovirals (Kredo *et al.*, 2009) for with sub therapeutic concentrations predispose to virological failure and high levels to adverse reactions.

## 1.3 Type II diabetes

Type II diabetes (TIIDM) (formerly called non-insulin-dependent or adult-onset) results essentially from the interaction of insulin resistance (IR) and insulin secretion deficiency (β-cell dysfunction) (Leahy, 2005). IR is defined as a decrease ability of insulin to be sensed by peripheral tissues (liver, muscle, fat) to stimulate glucose metabolism and/or inhibit hepatic glucose output. This condition will influence the glucose homeostasis (**Figure 1.2**). Several mechanisms as the serine phosphorylation of IRS-1 (Pratipanawatr *et al.*, 2001; Zhande *et al.*, 2002), excess glucosamine (Marshall *et al.*, 1991), defective mitochondria (Nyholm *et al.*, 1997; Kelley *et al.*, 2002) or alternate fatty acid effects (Saha and Ruderman, 2003; Schrauwen and Hesselink, 2004) have been proposed to explain this phenomenon. On the other hand, the impaired insulin secretion is characterized by a defect in the glucose-stimulated insulin secretion due dysfunction and/or reduction of a number of pancreatic islet β-cell (Kahn, 2000). The reason for this defect is still unknown; however there are many proposed pathological processes such as, glucose toxicity (Jonas *et al.*, 1999; Weyer *et al.*, 1999), beta-cell exhaustion (Greenwood *et al.*, 1976; Laedtke *et al.*, 2000), impaired pro-insulin biosynthesis (Olson *et al.*, 1998) and lipotoxicity (McGarry and Dobbins, 1999; Robertson *et al.*, 2004).

In addition to TIIDM, the combination of hyperglycemia and hyperinsulinaemia is also associated to other metabolic disorders including dyslipidemia, coronary heart disease and lipodystrophy and metabolic syndrome. Furthermore, chronic hyperglycemia plays even a significant role in many diabetes complications such as macrovascular (e.g. myocardial infarction, stroke) and microvascular damage (e.g. nephropathy, retinopathy, neuropathy) (Engelgau *et al.*, 2004).

In the patients with pre-diabetes is observed an increase level of glucose in the blood, but these levels are not significantly high to be considered as diabetes (< 126 mg/dl). Actually, the pre-diabetic individuals present a relative decrease in insulin sensitivity and an increase of insulin secretion by β-cell with the purpose of compensates the insulin resistance of peripheral tissues (Tabak *et al.*, 2012). If the insulin secretion falls, there is a glycemia worsening and TIIDM becomes eventually clinically manifest. It is noteworthy, that the prevalence of pre-diabetes is greater than TIIDM in worldwide (Gardete Correia *et al.*, 2012; Tabak *et al.*, 2012).

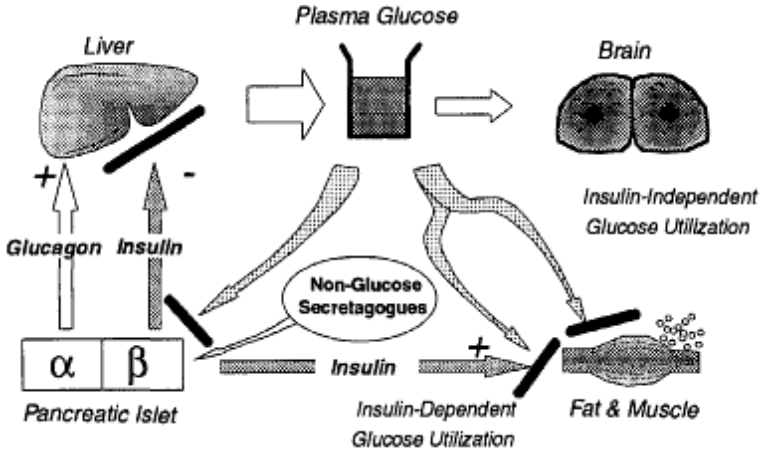


**Figure 1.2 - Representation of glucose homeostasis. In:** (Kahn, 1994)
This system depends upon a balance between glucose production (liver) and glucose uptake by peripheral tissues (fat and muscle, and brain). The hormones glucagon and insulin regulated this equilibrium. Insulin is released in order to reduce the glucose level in the blood whereas glucagon promotes hepatic glucose production.

## 1.4 Drug cycle and drug bioactivation

When administered orally, the drug penetrates the epithelial cells in the gastrointestinal tract and reaches the liver via hepatic portal vein, before entering in systemic circulation - a process called absorption. During the passage for gastrointestinal tract and the liver, a percentage of the drug may be lost due to the presence of enzymes responsible for biotransformation, the presence efflux drug transporters and excretion – this is known as first-pass effect (Le, 2012).

After its absorption, the drug is distributed by the several tissues. The rate and extent of this distribution is directly influenced by interactions of the drug with body components, which depend on both the physiochemical properties of the drug (e.g. lipid solubility, pK or molecular weight) and physiological parameters (e.g. pH, extent of plasma protein binding, blood flow or nature of the tissue) (Gibson and Skett, 2001)

Continuing in the drug cycle, the drug will be excreted mainly via the renal and biliar routes. However, in most of the cases, its conversion on polar compounds (more water-soluble), to facilitate their excretion, it is necessary. The drugs undergo a number of chemical reactions and this cycle phase is known as drug biotransformation or metabolism. For many drugs, this occurs in two phases: phase I and phase II reaction. Phase I reactions involve formation of a new or modified functional group or cleavage (oxidation, reduction, hydrolysis), while phase II involve conjugation with an endogenous substance (e.g. glucuronic acid, sulfate). The metabolites formed in the last phase are more polar and thus more readily excreted by the kidneys (in urine) and the liver (in bile).

In the phase I reactions, the most important player is the enzymatic system cytochrome (CYP) P450, which catalyzes the oxidation of many drugs. These enzymes can be induced or inhibited by many drugs or food components, which can consequently enhances the drug toxicity or reduce it therapeutic effect. In respect to the phase II phase, glucuronidation, sulfation and glutathione conjugation are the most prevalent reactions, which may occur directly on the parent compounds or, as normally happens, on the products of phase I (Xu *et al.*, 2005).

Although, the major intend of biotransformation is to inactivate and detoxify drugs that can harm the body, drugs upon biotransformation can also undergo bioactivation chemically reactive electrophilic or radical products that are potentially toxic by themselves or that produce oxidative stress (**Figure 1.3**) (Zhou *et al.*, 2005; Walsh and Miwa, 2011).

The electrophilic intermediates are capable of covalently modifying macromolecules (protein, nucleic acids, lipids), giving rise to the formation of drug-macromolecule adducts, which has long been associated with drug toxicity (Pumford *et al.*, 1997; Poirier *et al.*, 2000).

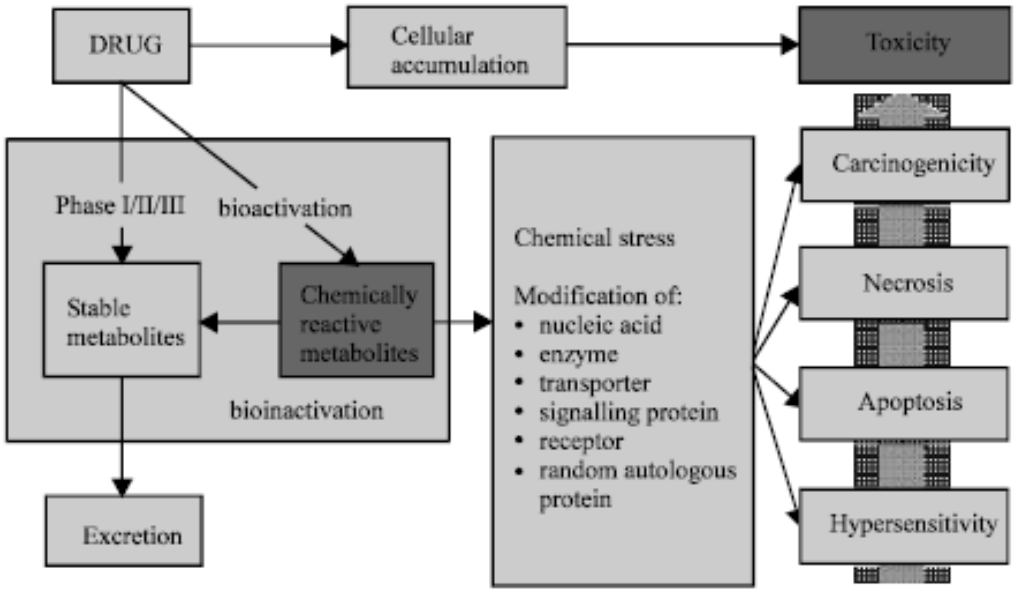


**Figure 1.3 - Toxicity related with drug biotransformation. In: (Williams *et al.*, 2012).**

### 1.4.1 Drug bioactivation and its implication on its toxicity

The liver is a major target organ for many toxic chemicals/drugs since it is the first site of drug metabolism and bioactivation. Moreover, the resulting reactive electrophiles could attack hepatic proteins readily because of their proximity (Abboud and Kaplowitz, 2007). The liver has an extraordinary ability to regenerate in response to cell damage.

The chemical-induced liver injury and repair model consist in three stages (**Figure 1.4**).

Upstream events in hepatocytes lead to exposure to electrophilic metabolites, which undergoes covalent binding after preferential depletion of glutathione (GSH). Then, upstream events promote intracellular stress, production of inflammatory mediators and mild injury which activates the innate immune system. Hepatoprotective mediators are secreted to restore cellular and organ structure and function and balance of pro- and anti-inflammatory responses, the interplay of which determines progression to severe injury or no injury (Chanda and Mehendale, 1995; Liu *et al.*, 2004).

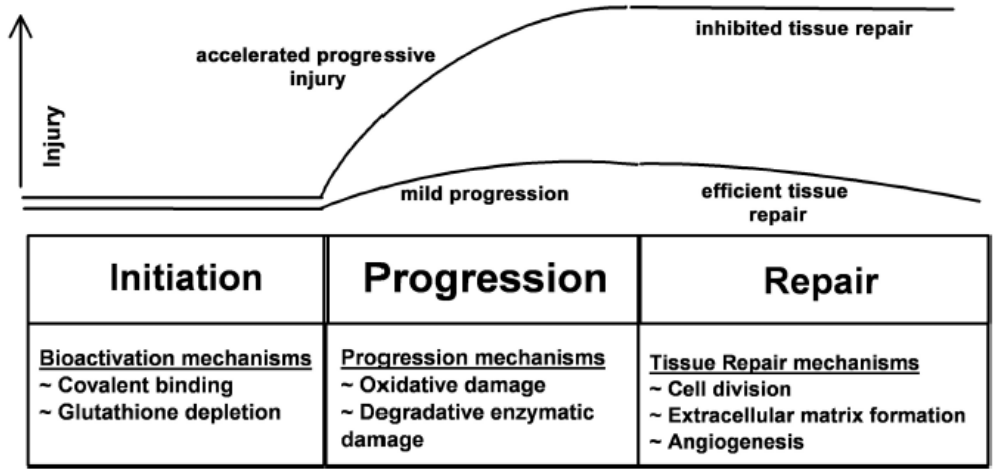


**Figure 1.4 - A 3-step mechanistic working model of hepatotoxicity: initiation, progression and tissue repair (or damage). In (Wang *et al.*, 2007).**

### 1.4.2 Diabetes and drug bioactivation

The compensatory liver tissue repair is compromised in diabetes (Sawant *et al.*, 2004; Sawant *et al.*, 2006; Wang *et al.*, 2007), as this pathology is associated to a sluggish tissue repair response. The high glucose concentration is implicated in inhibition of the hepatocyte proliferation (Caruana *et al.*, 1986; Chanda and Mehendale, 1995; Rao *et al.*, 1999). Also, the elevated levels of glucose and free fatty acid in plasma characteristic of diabetes are associated with an increasing production of free radicals and a diminishing of body antioxidant defences (Maritim *et al.*, 2003). In addition, glucose is also involved in the production of advanced glycation end-products (AGEs) which inactivate and modify enzymes as well as also promote free radical formation. Moreover, it has been proposed that AGEs might cause functional alterations in hepatocytes, leading to retarded cell proliferation and tissue repair (Cerami *et al.*, 1988; Chanda and Mehendale, 1995). The hyperglycemia impairs the antioxidant system, being glutathione (GSH) ,the most abundant low-molecular-weight thiol, one of the major and important scavengers of endogenous free radicals and drug electrophic toxic metabolites (Dickinson and Forman, 2002).

Whether diabetes development ends up influencing protein, lipids and carbohydrate metabolism and promoting oxidative stress, there is also an effect on the biochemical pathways involved in all drug

cycle steps (**Table 1.1**). While the effects of diabetes mellitus on drug PK and PD properties have been well described in experimental animal models; minimal clinical data exists for humans (Dostalek *et al.*, 2012).

The compromised liver repair on diabetes and initial drug bioactivation-based liver damage potentiate hepatotoxicity of drugs in diabetes context.

**Table 1.1 – The effect of TIIDM in drug PK.**

| PHARMACOKINETIC PRINCIPLES | EFFECT OF TIIDM | REFERENCES |
|---|---|---|
| **Absorption** | REDUCED intestinal transit time | (Jung *et al.*, 2003; Wu *et al.*, 2004; Triantafyllou *et al.*, 2007) |
| | REDUCED gastric mucosal blood flow | (Zhu, 1993) |
| | NO CONSENSUAL gastric emptying time:<br>- Decreased<br>- Unchanged<br>- Increased | (Keshavarzian *et al.*, 1987; Nowak *et al.*, 1995; Lyrenas *et al.*, 1997) (Kong *et al.*, 1999) (Frank *et al.*, 1995; Schwartz *et al.*, 1996) |
| **Distribution** | REDUCED protein binding capacity | (MacKichan, 2006) |
| | INCREASED free fraction of drugs | (Ruiz-Cabello and Erill, 1984; MacKichan, 2006) |
| **Biotransformation** | INCREASED enzymatic activity (e.g. CYP 2E1) | (Dostalek *et al.*, 2011) |
| | DECREASED enzymatic activity (e.g. CYP 3A4) | (Kotlyar and Carson, 1999; Brill *et al.*, 2012); (Dostalek *et al.*, 2011) |
| **Excretion** | INCREASED glomerular filtration rate | (Meeme and Kasozi, 2009) |

## 1.5 Nevirapine

Nevirapine (NVP) was the first NNRTI approved by the U.S. Food and Drug Administration (FDA) in 1996 (FDA, 1996). This first generation NNRTI is known for its efficacy in prevention of mother-to-child HIV-transmission, with the drug being commonly indicated for pregnant women and their children (Marseille *et al.*, 1999; Ades *et al.*, 2000; Lallemant *et al.*, 2004; AIDS, 2012). Moreover, NVP is described by having a favorable lipid and metabolic profile rendering it suitable for use in individuals with diabetes, dyslipidemia and other metabolic disorders (Ruiz *et al.*, 2001; Clotet *et al.*, 2003). Furthermore, NVP also stands out for being the most used antiretroviral in countries with limited resources due to its low cost (Ades *et al.*, 2000). These benefits represent the mean why NVP is globally the most prescribed antiretroviral.

Although the clinical benefits presented, NVP is associated with undesirable adverse effects. Among the clinical complications, the most common is skin rash and the most severe is hepatotoxicity. These reactions may lead to drug discontinuation or even be fatal (Cattelan *et al.*, 1999; Taiwo, 2006; De Lazzari *et al.*, 2008; Medrano *et al.*, 2008). Moreover, NVP can induce hepatocellular neoplasias in animal models (Anonymous 2009), and recent epidemiological data suggest an association between chronic NNRTI use and an increased incidence of non-AIDS-defining cancers in HIV-1-infected patients (Powles *et al.*, 2009).

The mechanisms underlying NVP toxicity are still not fully understood; however the current evidence is strongly consistent with a higher risk in women (Ho *et al.*, 1998; Antinori *et al.*, 2001; Bersoff-Matcha *et al.*, 2001) and a subjacent immune mediation (Martin *et al.*, 2005; Taiwo, 2006; Yuan *et al.*, 2011; Dong *et al.*, 2012). To allow for sex differences in immune hyper-reactivity, it is recommended that in cART-naive women NVP should be initiated only in those with a CD4+ cell count < 250 cells/mm$^3$, whereas in men this cut-off is 400 cells/mm$^3$ (Thompson *et al.*, 2010).

The administration of NVP starts with a 200mg dose for the first 14 days and then followed by a 400 mg daily dose. The lower dose in the first weeks is recommended by therapeutic schedule to minimize toxic events.

### 1.5.1 The Pharmacokinetic properties of nevirapine and its toxic reactions

As a highly lipophilic molecule, NVP is readily absorbed (greater than 90%) after oral administration. NVP is about 60% bound to plasma proteins, crosses the placenta and blood–brain barrier, and is found in breast milk (Mirochnick *et al.*, 2000).

The primary route of NVP biotransformation and elimination is represented by the combination of CYP P450 metabolism, glucuronide conjugation and urinary excretion (Riska *et al.*, 1999a). In humans, NVP biotransformation involves the generation of several hydroxylated phase I metabolites (at NVP positions 2, 3, 8 and 12) (**Figure 1.5**) (Erickson *et al.*, 1999; Riska *et al.*, 1999a).

The attempts to correlate NVP exposure and toxicity outcomes have so far been inconclusive (de Maat *et al.*, 2003; Regazzi *et al.*, 2003; Almond *et al.*, 2004; Kappelhoff *et al.*, 2005; Hall and MacGregor, 2007),which suggests that NVP biotransformation to reactive metabolites, rather than the levels of the parent drug, may be the basis of the drug's adverse effects. Whether or not and how these metabolites promote hypersensitivity reactions is unknown, but protein haptenation might be involved. In fact, upon bioactivation, these metabolites may generate electrophilic species (Chen *et al.*, 2008; Srivastava *et al.*, 2010) capable of binding covalently to proteins and other biomacromolecules (**Figure 1.5**) (Chen *et al.*, 2008; Antunes *et al.*, 2009; Wen *et al.*, 2009; Srivastava *et al.*, 2010; Caixas *et al.*, 2012; Sharma *et al.*, 2013). The involvement of phase II reactions, and particularly the sulphotransferase (SULT)-mediated formation of 12-sulphoxy NVP from 12-hydroxy NVP, has recently gained increased support as a major player in this context (Chen *et al.*, 2008; Antunes *et al.*, 2009; Wen *et al.*, 2009; Antunes *et al.*, 2010; Meng *et al.*, 2013; Sharma *et al.*, 2013), with protein adducts from this pathway having already been detected in patients on therapeutic doses of NVP (Caixas *et al.*, 2012; Meng *et al.*, 2013).

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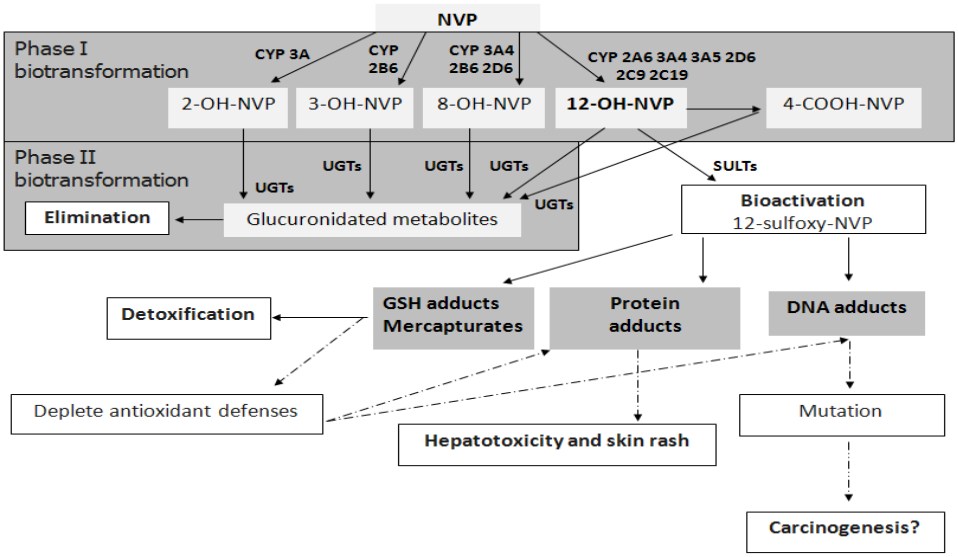


**Figure 1.5 - NVP biotransformation, disposition and proposed bioactivation pathways. In: (Marinho *et al.*, 2013).**

NVP (NVP) is metabolized by several isoforms of cytochrome P450 (CYP) yielding several phase I metabolites: 2-hydroxy NVP (2-OH NVP), 3-hydroxy NVP (3-OH NVP), 8-hydroxy NVP (8-OH NVP), and 12-hydroxy NVP (12-OH NVP) (Riska *et al.*, 1999a). 12-OH NVP metabolite is further oxidized by CYP450 to yield 4-carboxy-NVP (4-COOH-NVP) (Chen *et al.*, 2008). The phase I NVP metabolites undergo extensive glucuronidation via UDP-glucuronosyltransferase (UGT), which represents a major pathway of NVP elimination (Riska *et al.*, 1999a). The bioactivation of 12-OH NVP by sulfotransferases (SULTs) can generate 12-sulfoxy NVP, a reactive metabolite that binds covalently to proteins and DNA (Antunes *et al.*, 2008; Caixas *et al.*, 2012; Meng *et al.*, 2013). The formation of DNA adducts could explain the increased incidence of non-AIDS defining cancers among HIV-infected patients treated with non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Powles *et al.*, 2009). Also, the formation of adducts with proteins could explain the NVP-associated adverse reactions, hepatotoxicity and skin rash (Yuan *et al.*, 2011). The presence of glutathione (GSH) adducts and mercapturates in patients and animal models treated with NVP has also been detected (Srivastava *et al.*, 2010).

## 1.6 Rational, aim and work plan

**What we know?**

- The number of HIV/infected patients with pre-diabetes is increasing;
- The NNRTIs efavirenz and nevirapine based cART are recommend first line choice;
- NVP is a protective factor for pre-diabetes;
- Diabetes influences drug pharmacokinetics and toxicokinetics;
- NVP is a drug with a narrow therapeutic window: sub therapeutic NVP concentrations are associated with therapeutic failure and resistance development and higher NVP concentrations predispose for toxicity;
- NVP is toxic to the skin and liver and the first steps mechanism underlying it involve bioactivation of its phase I metabolite 12-OH NVP and protein adducts formation;
- Glutathione is a defense system for adducts formation and hepatotoxicity prevention, which levels are decreased in diabetes.

**What we need to know?**

- What is the effect of pre-diabetes on NVP pharmacokinetics?
- What is the effect of pre-diabetes on NVP metabolites profile?
- What are the effects of NVP on glycemia and insulin sensitivity?
- What are the effects of NVP in thiols antioxidant system?

*Generally, is expected to give new insights on the applicability of NVP in HIV infected patients with pre-diabetes, in the view of NVP pharmacokinetic changes induced by diabetes and the consequent effect on its efficacy and toxicity.*

**The specific aims of this work are:**

- To evaluate the **pre-diabetes effect** on:
  - Plasmatic and hepatic NVP bioavailability;
  - NVP biotransformation, phase I metabolites (12-OH NVP and 2-OH NVP) bioavailability and metabolite profile.

- To evaluate the **NVP effect** on:
  - Fasting glycemia, insulin sensitivity and mean arterial pressure;
  - Antioxidant status evaluation via thiol (homocysteine, cysteine, cysteinyl-glycine and glutathione) content measurement.

**Work Plan**

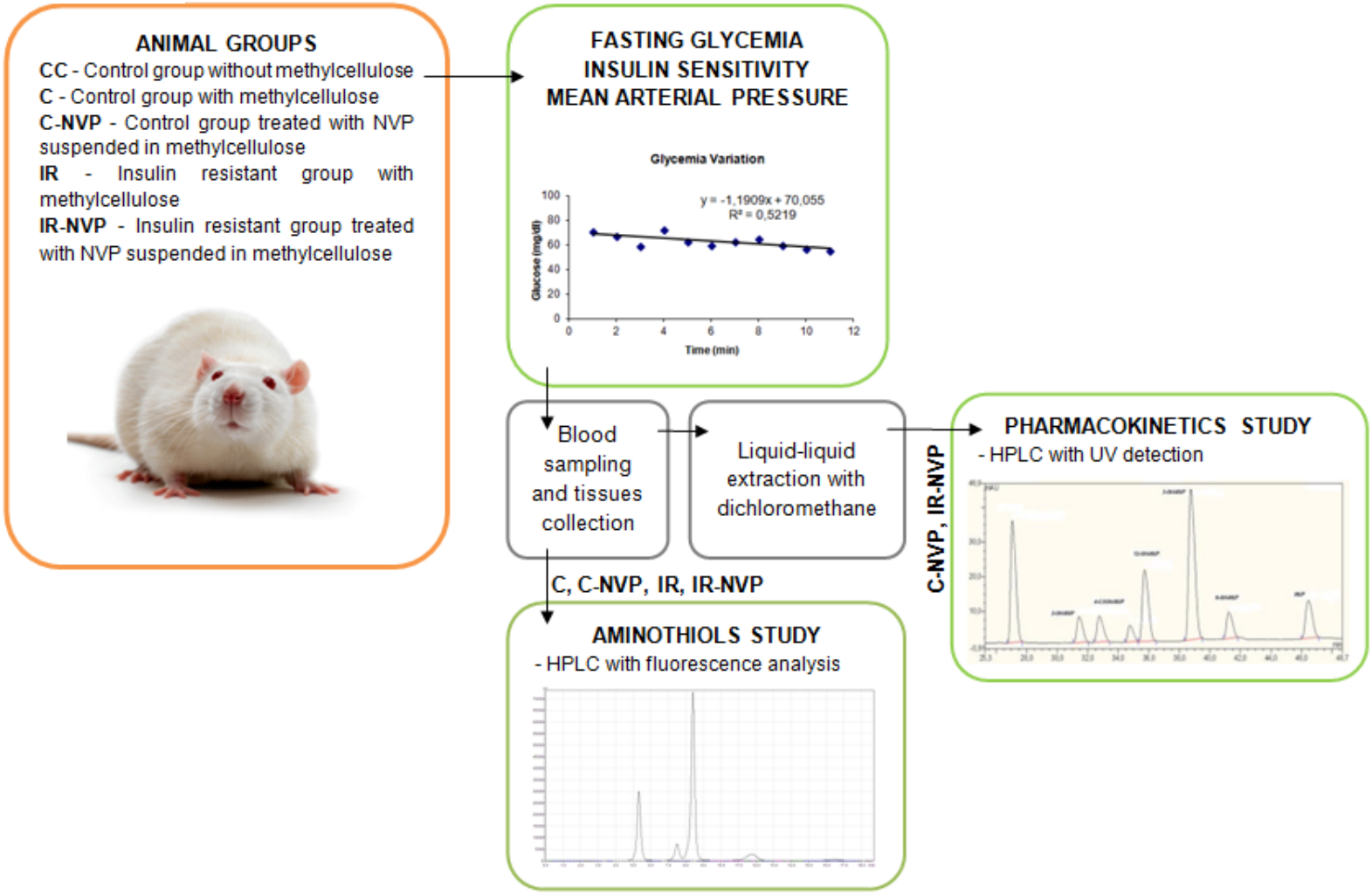


**Figure 1.6 – Illustrative summary of work plan.**

# 2. Materials and Methods

## 2.1 Animals maintenance and study group

The animals were females obtained from the vivarium of the NOVA Medical School (FCM), NOVA University (UNL), where were kept under temperature and humidity control (21±1 ºC; 55±10% humidity) with 12/12h light/dark cycles and fed a standard chow (7.4% fat + 75% carbohydrate (4% sugar) + 17% protein; SDS diets RM1 (*Probiológica*, Sintra, Portugal)).

All procedures were followed in accordance to the *European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends* (2010/63/ EU) and were previously approved by *Ethic Committee for Animal Care and Use* at FCM-UNL.

Five groups of rats aged between 11 to 20 weeks (0.22-0.30 kg) were used: Control group without methylcellulose (CC); Control group with methylcellulose (C); Control group treated with NVP solved in methylcellulose (C-NVP); Insulin resistant group with methylcellulose (IR); Insulin resistant group treated with NVP solved in methylcellulose (IR-NVP).

NVP (*Cipla*, Maharashtra, India) was administered at a dose of 40 mg/kg/day in a 0.5% methylcellulose solution (MC) in the drinking water bottles, during 8 days. Methylcellulose was used to facilitate dissolution of NVP (Mitchell *et al.*, 2003).

The insulin resistant animal model (IR group) used was the high sucrose (HSu) rat, a lean model of combined insulin resistance and hypertension (Ribeiro *et al.*, 2005; Conde *et al.*, 2012), which was obtained by submitting the animals to 35% of sucrose *GPR Rectapur* (VWR, Belgium) in drinking water during 28 days (**Figure 2.1**).

In IR-NVP group, NVP was administered during 8 days after the 28 days of HSu diet. C and IR groups were also administered with the vehicle 0.5% methylcellulose (MC) (*Sigma-Aldrich Corporation*, St. Louis, MO, USA) in drinking water (**Figure 2.1**).

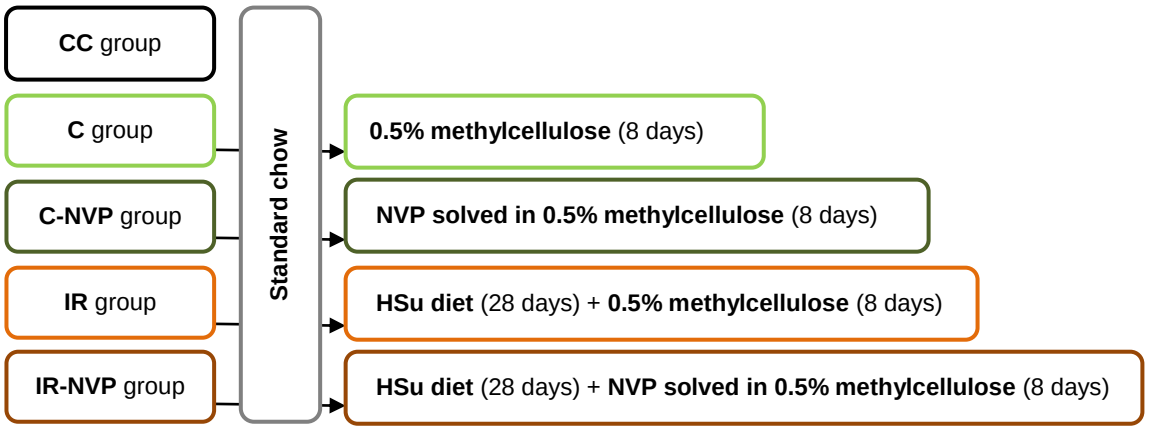


**Figure 2.1 - Animal maintenance and study groups.**
CC – Control group without methylcellulose; C – Control group with methylcellulose; C-NVP – Control group treated with NVP solved in methylcellulose; IR – Insulin resistant group with methylcellulose; IR-NVP – Insulin resistant group with NVP solved in methylcellulose; HSu – High sucrose diet; NVP – Nevirapine.

Drinking water daily intake was measured to monitor nevirapine and/or sucrose ingestion, depending on group. Animal body weight was assessed twice per week.

On the last day of the experimental protocol, the rats were fasted overnight and allowed free access to water or nevirapine solution, according to the groups.

After that, the animals were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg) and were transferred to a heating pad to maintain body temperature at 37.5±0.5 ºC throughout the experiment.

### 2.1.1 Blood sampling and liver collection

After insulin sensitivity and mean arterial pressure (MAP) measurements (**Section 2.3.2.2**), blood was collected by heart puncture into EDTA pre-coated tubes. The plasma was obtained by blood centrifugation (*Sigma Laborzentrifugen 2K15*, Osterode am Harz, Germany) at 3000 *g* and 4 ºC*,* for 10 minutes. The liver was extracted and cryopreserved at -80 ºC, until posterior study.

## 2.2 Pharmacokinetic assessment

### 2.2.1 Drugs and reagents

The ammonium acetate was obtained from *Merck S.A.* (Darmstadt, Germany) and acetonitrile from *Lab-Scan, analytical sciences* (Gliwice, Poland). Nevirapine was obtained from *Cipla* (Maharashtra, India) and the 2-OH, 3-OH, 8-OH and 12-OH NVP metabolites were synthesized as previously described (Antunes *et al.*, 2010) and used as standards for HPLC quantification. The other reagents used were mainly purchased from *Sigma-Aldrich Corporation* (St. Louis, MO, USA).

### 2.2.2 Preparation of calibration solutions

Stock solutions of NVP and its phase I metabolites (2-OH, 12-OH, 3-OH and 8-OH NVP) were prepared by dissolution in a water:methanol mixture (50:50 v/v). Different standard solutions were prepared by solving stock solutions (50 µL) in plasma (950 µL) and liver homogenate in phosphate buffered saline (PBS) 1:10 proportion (950 µL) from control rats, to obtain a calibration curve covering the concentration range from 0.005 to 10 mg/L. Standard solutions were stored at -80 ºC after handling.

### 2.2.3 Sample pre-treatment

A liquid-liquid extraction procedure was performed. Briefly, dichloromethane was added to *Wistar* rat plasma (250 µL) in a proportion of 1:10 and liver homogenate (12 mL) in a proportion of 1:2. The organic phase was recovered and the aqueous phase was re-extracted. Two and three extractions were performed to plasma and liver, respectively. The total organic phase obtain were collected and dried under vacuum (*Centrivap micro IR, Labconco* (Kansas City, USA)) at 60 ºC. The solid residues were reconstituted in 150 µL of water:methanol (50:50) mixture, centrifuged (13 000 *g*, 3 minutes, 4 ºC) for suspension removal and insert into high-performance liquid chromatography (HPLC) vials.

### 2.2.4 High-performance liquid chromatography (HPLC) analysis

HPLC analyses of nevirapine and its metabolites was conducted on an Agilent 1100 Series system (*Agilent Technologies* (Santa Clara, CA, USA)) using a reversed-phase Luna C18 (2) column (250 mm x 4.6 mm; 5 µm; Phenomenex, Torrance, CA, USA). The mobile phase—10% acetonitrile in 15 mM ammonium acetate buffer, pH 4—was delivered at a flow rate of 0.8 mL/min for 90 min; the flow rate was then increased to 1.5 mL/min over 5 min and maintained at this value for an additional period of 19 min. The column temperature was 40 ºC, the injection volume was 100 µL and UV absorbance was monitored at 254 nm.

The chromatographic peaks occurred at 34 min for 2-OH NVP, 52 min for 12-OH NVP, 69 min for 3-OH NVP, 85 min for 8-OH NVP and 109 min for NVP (**Figure 2.2**).

The lower limit of quantification of the method was 10 ng/mL for each analyte.

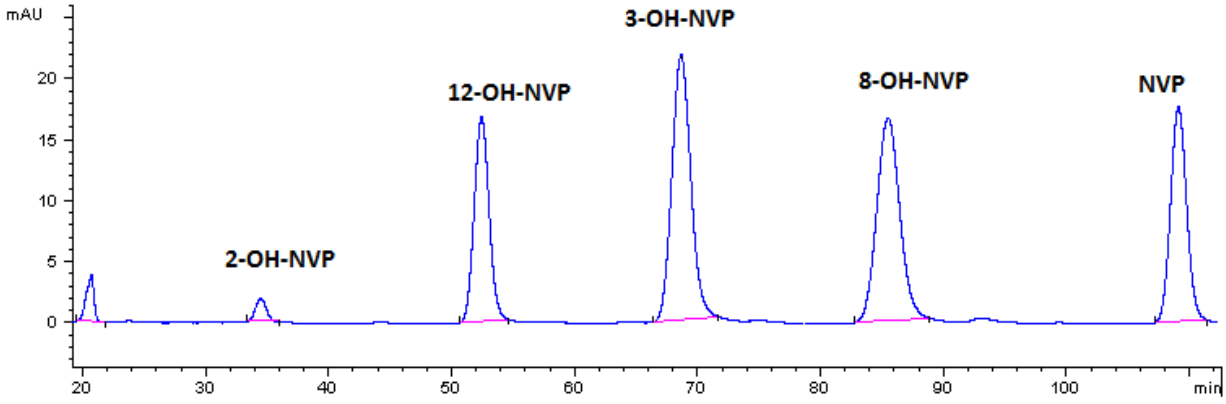


**Figure 2.2 - Representative chromatogram of a mixture of nevirapine (NVP) and its phase I metabolites under the conditions described previously.** The analyte concentrations were 2.5ng/mL.

## 2.3 Fasting glycemia, insulin sensitivity and mean arterial pressure assessment

### 2.3.1 Drugs and reagents

Insulin is commercially available as *Humulin Regular® Lilly* (Algés, Portugal) in a concentration of 100 UI/mL.

### 2.3.2 Measurement of insulin sensitivity

The insulin tolerance test (ITT) was used to measure insulin sensitivity**.** The ITT is one of the earliest developed methods to assess insulin sensitivity *in vivo* and provides an estimate of overall insulin sensitivity, correlating well with the 'gold standard' hyperinsulinaemic–euglycaemic clamp (Monzillo and Hamdy, 2003; Conde *et al.*, 2012). This method consists in the administration of an intravenous insulin bolus of 0.1 U/kg body weight after an overnight fast, followed by measuring the decline in plasma glucose concentration over 15 min at 1 min intervals. The constant rate for glucose disappearance ($K_{ITT}$) was calculated using the formula $0.693/t_{1/2}$. Glucose half-time ($t_{1/2}$) was calculated from the slope of the least square analysis of plasma glucose concentrations during the linear decay phase. Blood samples were collected by tail tipping and glucose levels were measured with a glucometer (*Abbott Diabetes Care*, Amadora, Portugal) and test strips (*Abbott Diabetes Care*, Amadora, Portugal).

### 2.3.3 Measurement of blood pressure

To measure mean arterial pressure (MAP), the femoral artery was cannulated under a dissection microscope and the catheter was connected to a pressure transducer (model 603; *HSE-HA GmgH*, *Harvard Apparatus*, Madrid, Spain) and pressure amplifier (*Plugsys Housings, modelo 603, HSE-HA GmgH, Harvard Apparatus*, Madrid, Spain). MAP data were acquired with *HSE-Harvard PULMODYN W* software (*Harvard Apparatus*, Madrid, Spain).

## 2.4 Antioxidant assessment in rat plasma and liver: the aminothiols.

### 2.4.1 Reagents

Sodium hydroxide was purchased from *Merck S.A.* (Darmstadt, Germany). The ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F) was obtained from *Santa Cruz Biotechnology, Inc.* (Heidelberg, Germany). Stock solutions of cysteine (Cys), homocysteine (HCys), cysteinyl-glycine

(Cys-Gly), glutathione (GSH) and N-acetylcysteine (NAC) as well the most of the reagents were purchased from *Sigma-Aldrich Corporation* (St. Louis, MO, USA).

### 2.4.2 Aminothiol measurement in plasma

The method for sample preparation was adapted from (Nolin *et al.*, 2007).

#### 2.4.2.1 Standard preparation

Stock solutions were prepared by solving each thiol in water. These solutions were used to prepare standard solutions in PBS in the following concentration range 5 – 800 µM for Cys, 0.625 – 100 µM for HCys, 1.25 – 200 µM for Cys-Gly, and 0.313 – 50 µM for GSH and NAC.

#### 2.4.2.2 Sample preparation

Samples were prepared following three main steps: thiol reduction, protein precipitation and derivatization. In first step, 10 µL of Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) 100 g/L were added to 100 µL of plasma sample, briefly vortex-mixed and incubated at room temperature for 30 min. Subsequently, trichloroacetic acid (TCA) 100 g/L with EDTA 1 mM was added, briefly vortex-mixed and centrifuged at 13000 *g*, for 10 min, at 4 ºC and the supernatant was collected. Afterward, the following solutions were added to 50 µL of supernatant: 10 µL of NaOH 1.55 M, 125 µL of borate buffer 0.125 M, pH 9.5 with EDTA 4 mM and 50 µL of ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F) 1 g/L in borate buffer 0.125 M with EDTA 4 mM. Samples were briefly vortex-mixed and incubated for 1 h, at 60 ºC, in the dark. Finally, the samples were cooled on ice and protected from light until analyses.

#### 2.4.2.3 High-performance liquid chromatography (HPLC) with fluorescence analysis.

Total of 10 µL of samples were injected onto HPLC. During the analytical runs the samples were kept in the refrigerated autosampler at 8 ºC. This quantification was performed in *Schimadzu* (Kyoto, Japan) system, consisting of a LC 9-A solvent delivery pump, a 7725i injector, a RF-10AXL fluorescence detector and a CTO-10AS VP column oven. It was used a *LiChrospher* 100 RP-18 (250 x 4 mm; 5 µm) column protected by a *LiChrospher* 100 RP-18e (4x 4 mm; 5 µm) guard-column, both from *Merck* (New Jersey, USA) at 29 ºC. The detector was set at excitation and emission wavelengths of 385 nm and 515 nm, respectively. Data acquisition and processing were performed on the Shimadzu Class VP 7.X software. Isocratic elution method was used with a mobile phases consisted of acetate buffer 0.1 M pH 4.5 - methanol 99:1 (v/v) in a flow rate of 0.8 mL/min. Analysis duration was 20 min. The chromatographic peaks occurred at 6 min for Cys, at 8 min for HCys, at 9 min for Cys-Gly, at 13 min for GSH and at 17 min for NAC (**Figure 2.3**).

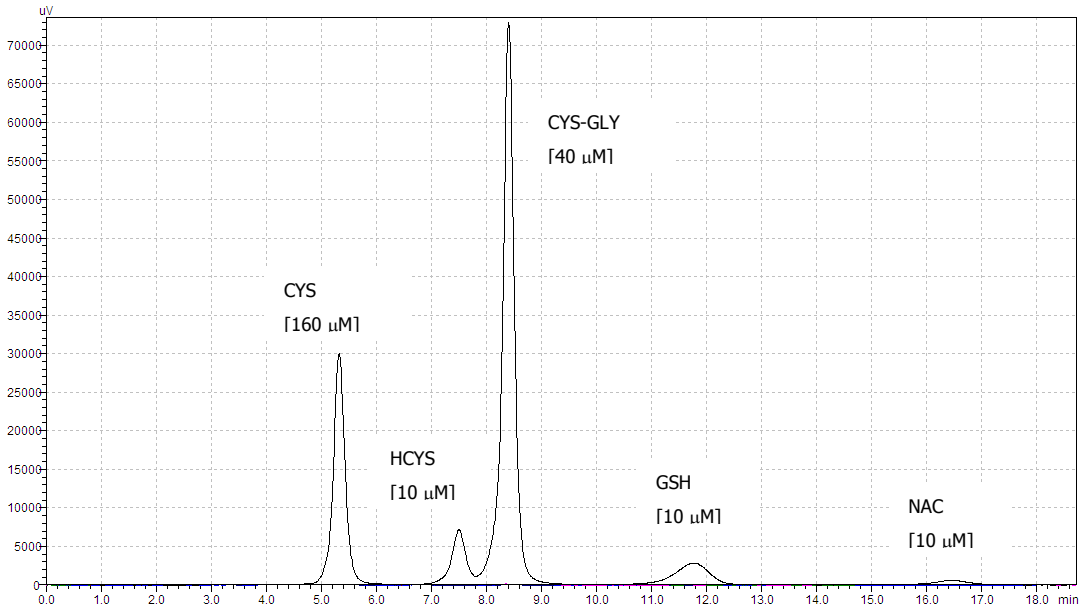


**Figure 2.3 - Representative chromatogram of aminothiols.**
Cys - cysteine, HCys – homocysteine, Cys-Gly - cysteinyl-glycine, GSH – glutathione, NAC - N-acetylcysteine (NAC)

### 2.4.3 Aminothiol measurement in liver

The method for sample preparation was adapted from (Nolin *et al.*, 2007).

#### 2.4.3.1 Standard preparation

Standards of thiols were prepared accordingly the method described for plasma but with different concentration ranges: 1.25 – 200 µM for Cys, 0.16 – 25 µM for HCys, 0.31 – 50 µM for Cys-Gly, 5 – 800 µM for GSH and 0.0781 – 12.5 for NAC.

#### 2.4.3.2 Sample preparation

For preparation of liver samples an additional step towards thiol extraction, prior to aminothiol reduction, was necessary. For that, 250 µL of liver homogenate sample (prepared as described in **2.2.3 Section**) were centrifuged in an *Amicon* tube of 10 000 MWCO at 3800 *g,* for 20 min, at 4º C before thiol reduction with TCEP 100 g/L.

#### 2.4.3.3 High-performance liquid chromatography (HPLC) with fluorescence analysis.

The quantification of aminothiols in liver was performed by the same HPLC method conditions previously described in **2.4.2.3. Section**.

## 2.5 Statistical data analysis

The statistical analysis was performed using Graph Prism 5.0 (*GraphPad software Inc*.). The data were presented with mean ± standard error of the mean (SEM). The *t-test* and *Analysis Variance* (ANOVA) test were used to explore differences among groups. Potential relationships between the variables under analysis were explored using *Pearson's* and *Spearman's tests.*

# 3. Results

## 3.1 Effect of pre-diabetes on nevirapine disposition and biotransformation

The profile of NVP and its phase I metabolites 12-OH and 2-OH NVP, in plasma and liver, for C-NVP and IR-NVP is represented in **Figure 3.1**.

A decline in the plasma concentration of NVP and 12-OH NVP was observed in the IR-NVP group (**Panel A**). The same effect was found in the liver (**Panel B**). Although, the concentration differences for 2-OH NVP were failed to be proved in both liver and plasma. In addition, the profile of metabolites has also changed. While 12-OH-NVP is the major metabolite in C-NVP group, this pattern is lost in IR-NVP animals.

In **Figure 3.2** is represented the metabolite ratio NVP/12-OH NVP and NVP/2-OH NVP in C-NVP and in IR-NVP. In plasma only the NVP/2-OH NVP ratio was lower and in liver both ratios decreased in IR-NVP.

## 3.2 Effect of nevirapine on fasting glycemia, insulin resistance and blood pressure in pre-diabetic rats

A control group of animals without treatment and without methylcellulose (CC) was included to explore the effects of MC on animals MAP, fasting glycemia and insulin sensitivity and no effects of MC were found.

Additionally MAP was similar across groups (**Figure 3.3 Panel A**). The IR-NVP group is not represented because due to a software crash the files were lost**.**

The influence of NVP on fasting glycemia and insulin sensitivity is represented in **Figure 3.3 Panel B** and **C** respectively. In C-NVP, the drug did not modify significantly fasting glycemia. However, when NVP was administered after the 28 days of HSu diet, it was able of decrease it. The ITT constant ($K_{ITT}$) was not different across groups.

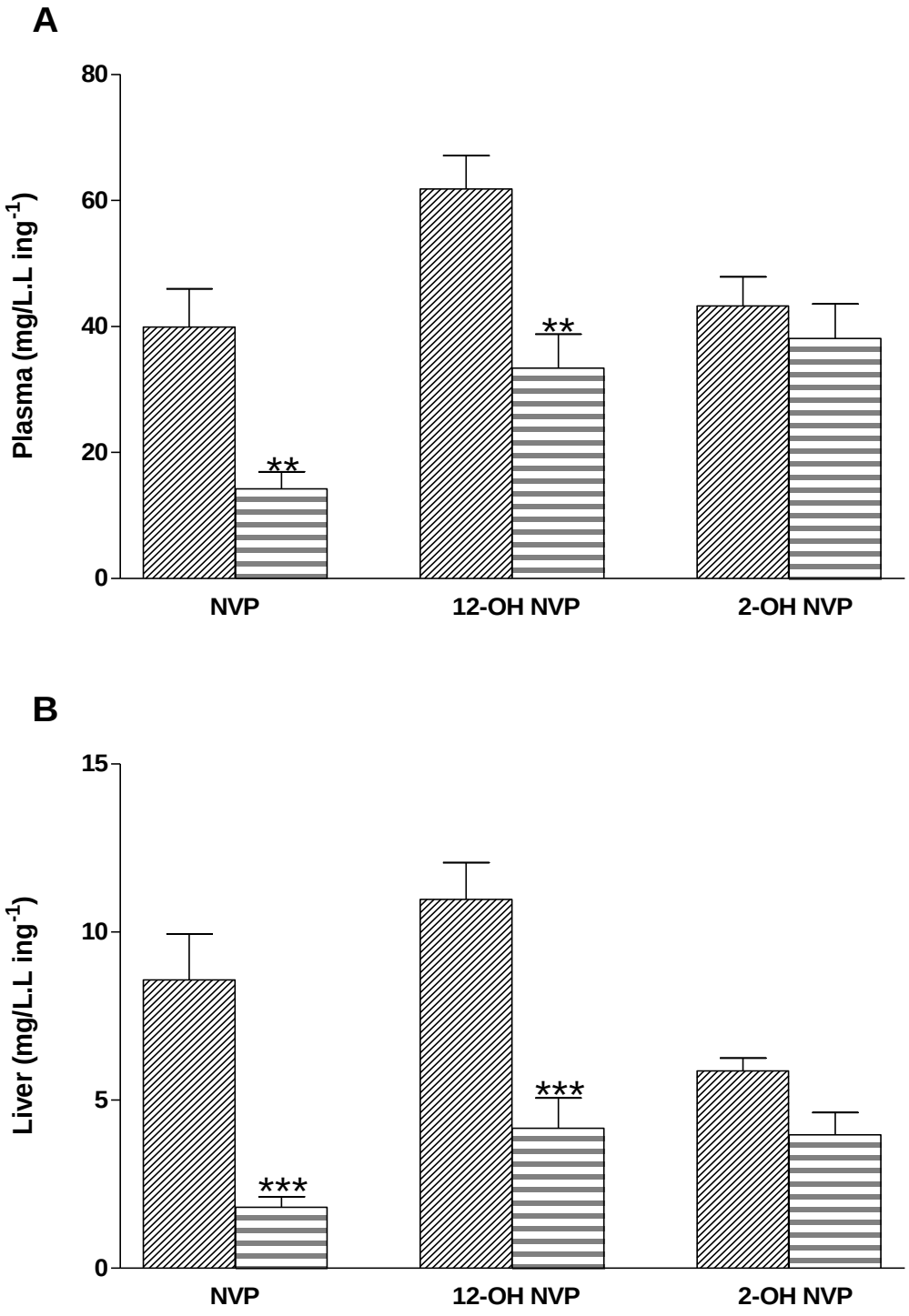


**Figure 3.1 - Nevirapine and its phase I metabolites in plasma (A) and liver (B).**
( ▨ ) Control group treated with NVP (C-NVP) (*n* = 9 *per* group); ( ▤ ) Insulin resistant group treated with NVP (IR-NVP) (*n* = 6, *per group*). Comparisons presented reflect differences between groups for the same analyte. ***P*<0.01, ****P*<0.005 (*One-way ANOVA* with *Bonferroni's* Multiple Comparison test). NVP – nevirapine; 12-OH NVP – 12-hydroxy nevirapine and 2-OH NVP – 2-hydroxy nevirapine.

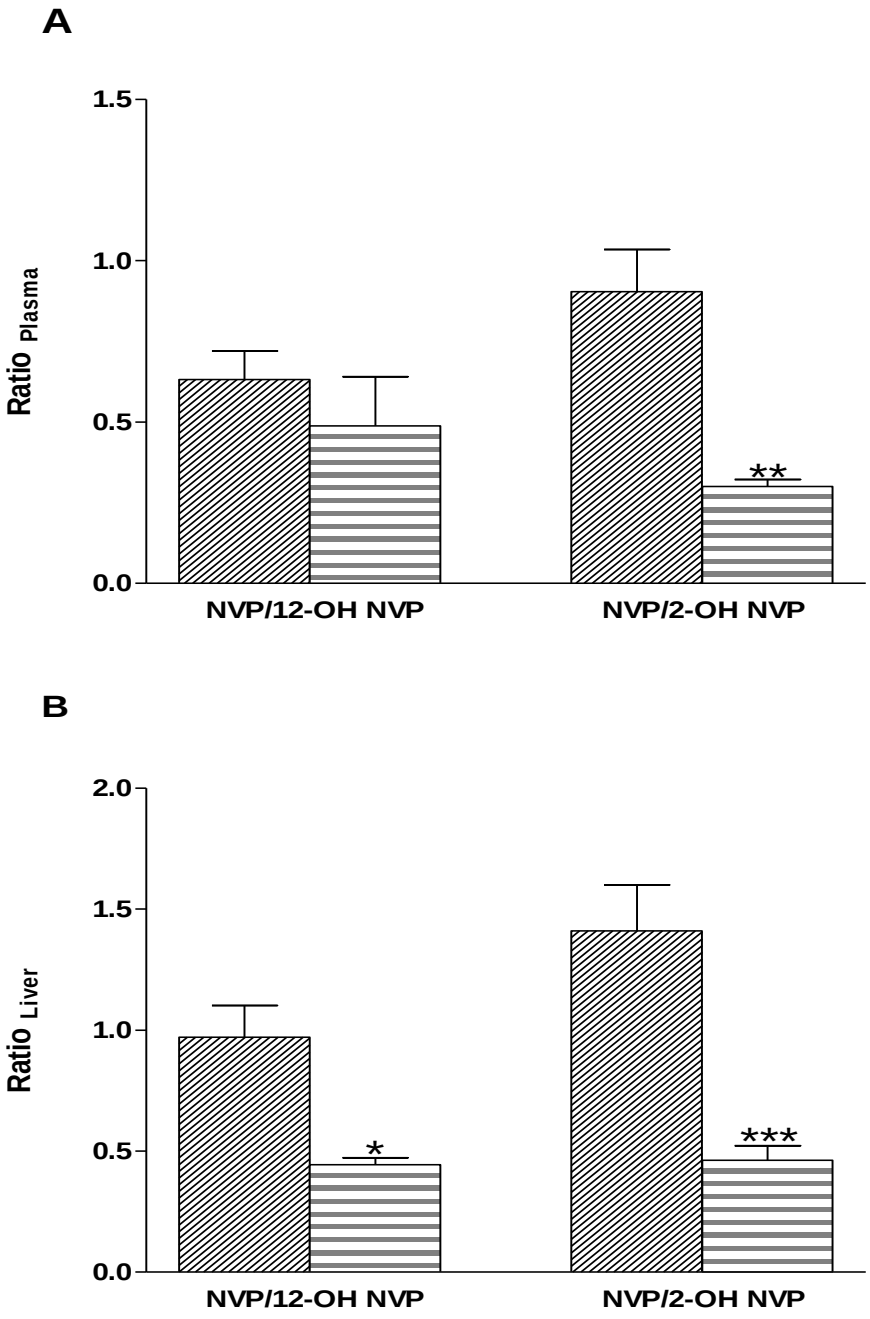


**Figure 3.2 - Nevirapine/12-hydroxy nevirapine (NVP/12-OH NVP) and Nevirapine/ 2-hydroxy nevirapine (NVP/2 OH NVP) ratios in plasma (A) and liver (B).**

(▨) Control group treated with NVP (C-NVP) (*n* = 9 *per* group); (▤) Insulin resistant group treated with NVP (IR-NVP) (*n* = 6 *per* group). Comparisons presented reflect differences between groups for the same analyte. **P*<0.05, ***P*<0.01, ****P*<0.005 (*One-way ANOVA* with *Bonferroni's* Multiple Comparison test).

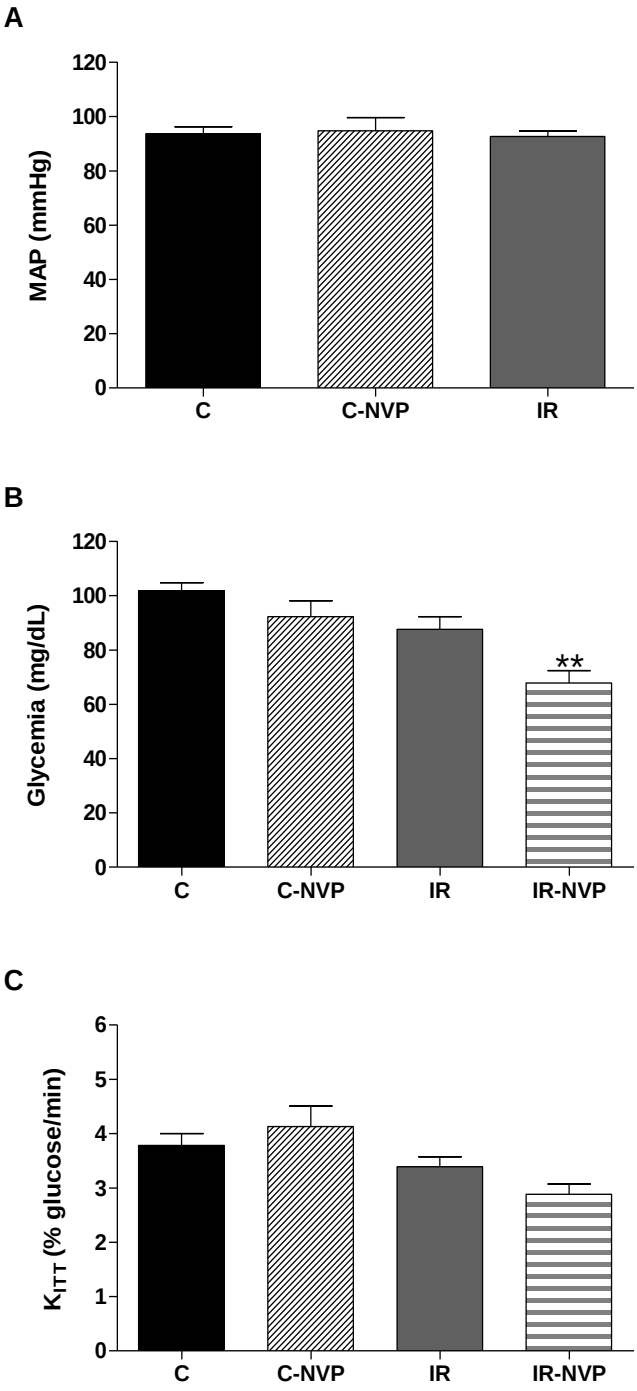


**Figure 3.3 - Effect of NVP intake in mean arterial pressure (A), basal glycemia (B) and insulin sensitivity (C).**

( ) Control group (C) (*n* = 10); ( ) control group treated with NVP (C-NVP) (*n* = 4); ( ) insulin resistant group (IR) (*n* = 4); ( ) insulin resistant group with NVP (IR-NVP) (*n* = 6)

The vertical bars represent mean ± SEM. Comparisons presented reflect differences between IR and IR-NVP group: ***P*<0.01 (*One-way ANOVA* with *Bonferroni* multiple comparison test).

MAP – mean arterial pressure; $K_{ITT}$ – constant rate for glucose disappearance.

## 3.3 Effect of nevirapine on thiol system

The total aminothiol concentration (tHCys, tCys, tCys-Gly and tGSH) found in plasma of each group (C, C-NVP, IR, IR-NVP) is represented in **Figure 3.4**.

For total homocysteine only insulin resistant effect was considered significant with higher levels in IR animals. No effect of treatment was found. There were no differences in total cysteine across groups. About tGSH were not found difference too. tCys-Gly plasma levels were higher elevated in insulin resistance animals but NVP treatment decreased these levels.

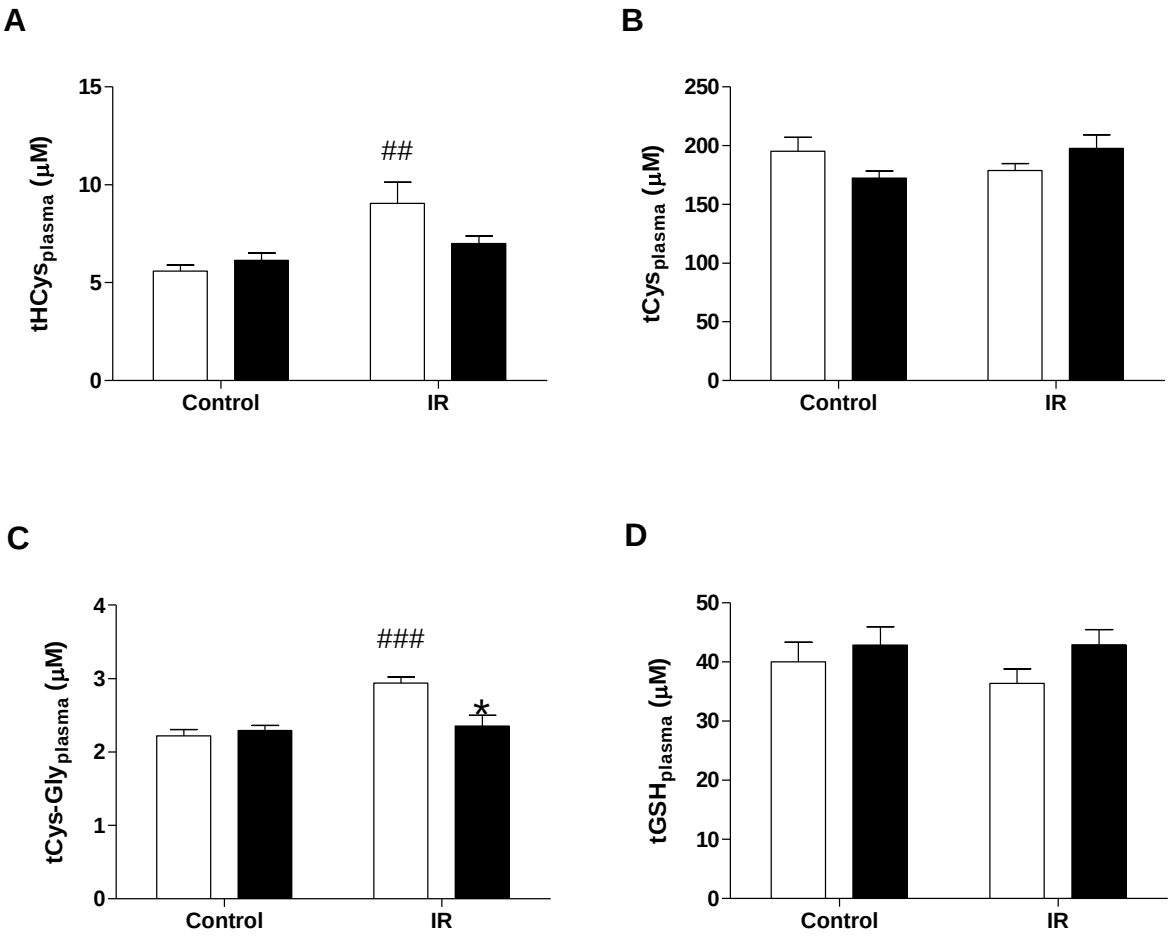


**Figure 3.4 - Plasma concentrations of Total Aminothiol**
(☐) Not treated (*n* = 6 *per* group); (■) Treated (*n* = 6 *per* group). A – total homocysteine level (tHCys); B – total cysteine level (tCys); C – total cysteinyl-glycine level (tCys-Gly); D – total glutathione level (tGSH). Concentration of aminothiols (mean ± SEM) is expressed in µM. Data are statistically different between control and insulin resistant groups (C and IR) $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$(*Two-way ANOVA* with *Bonferroni's* Multiple Comparison test). Data are statistically different between not treated and treated groups $^{*}P<0.05$ (*Two-way ANOVA* with *Bonferroni's* Multiple Comparison test).

For the liver, no significant differences across groups were found to tHCys, tCys and tCys-Gly (**Figure 3.5 Panel A, B and C**). For total glutathione only insulin resistant effect was considered significant with higher lower in IR animals (**Figure 3.5 Panel D**).

Also, tNAC levels were detected on liver (data don't shown). However it was not detected in all animals: four in control (C; C-NVP) and in insulin resistant not treated (IR), and two animals in insulin resistant NVP treated group (IR-NVP). While, pre-diabetes seems not have influence on these levels the effect of treatment reduced tNAC content (**Annex 1**).

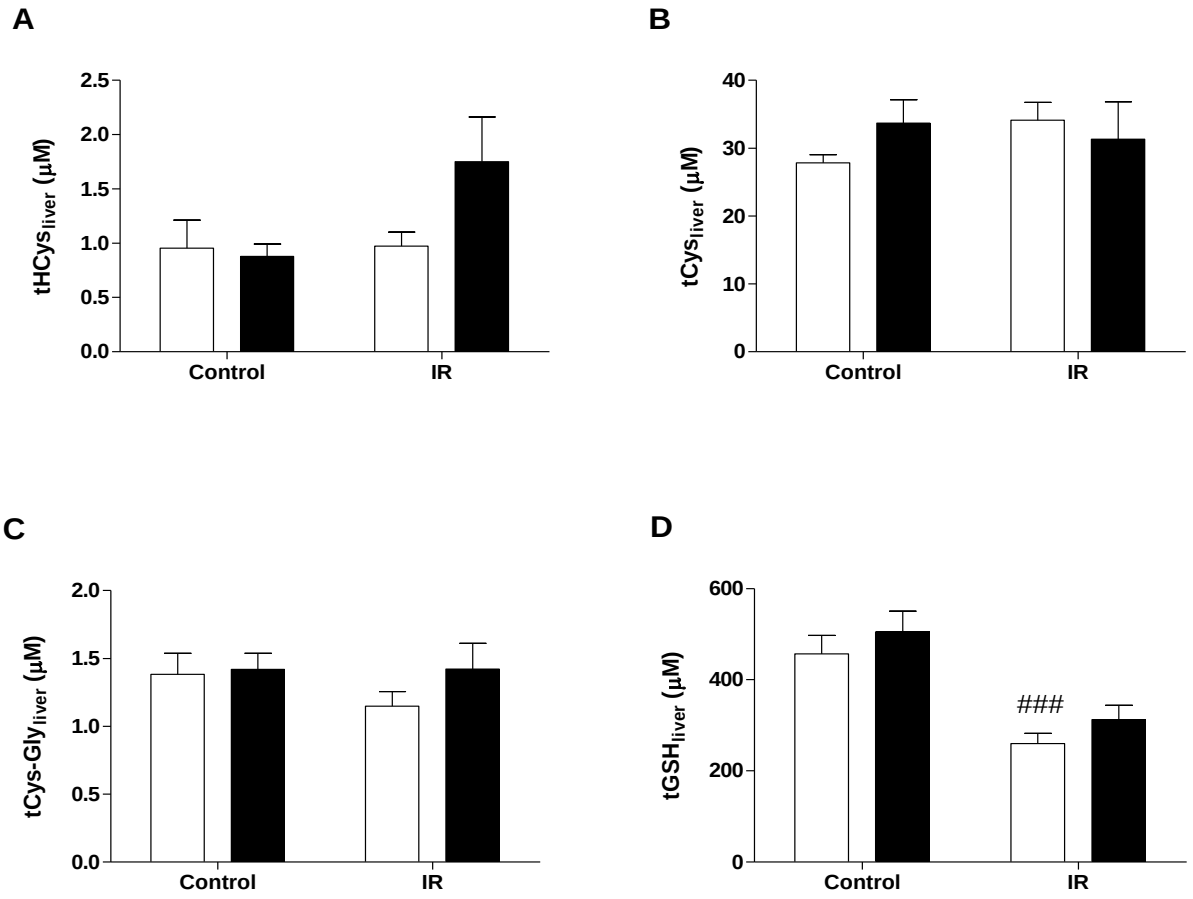


**Figure 3.5 - Liver concentrations of Total Aminothiol**
(☐) Not treated (*n* = 6 *per* group); (■) Treated (*n* = 6 *per* group). A – total homocysteine level (tHCys); B – total cysteine level (tCys); C – total cysteinyl-glycine level (tCys-Gly); D – total glutathione level (tGSH). Concentration of aminothiols (mean ± SEM) is expressed in µM. Data are statistically different between control and insulin resistant groups (C and IR) ###P<0.001(*Two-way ANOVA* with *Bonferroni's* Multiple Comparison test).

# 4. Discussion

In the present work an influence of a pre-diabetic status on NVP PK was shown: NVP and its phase I metabolites 12-OH NVP and 2-OH NVP presented changes in their disposition and the metabolite profile pattern of NVP was changed. Moreover, NVP has no effect on fasting glycemia and insulin sensitivity in a non-diabetic context indicating that it can be a good option for pre-diabetes prevention, while in a pre-diabetic perspective is associated with a benefic effect on fasting glycemia. The drug has no effect on mean arterial pressure and insulin resistance. Moreover, nevirapine is associated with a lower degradation of total glutathione. So far, there are no similar studies reported in literature, being this data pivotal in this area.

The management of HIV-infection is gradually expanding to include the chronic complications associated with this infection and the undesirable effects associated with antiretroviral drugs. For the past 20 years, researchers and clinicians have reported changes in fasting glycemia and insulin sensitivity in this population. Although, most of the data generated focus on the first available antiretroviral as stavudine, didanosine, zidovudine or indinavir (Mulligan *et al.*, 2000; Murata *et al.*, 2000; Blumer *et al.*, 2008; De Wit *et al.*, 2008) that, due to its toxic profile, have been substituted for safer antiretroviral in first-line therapy. Moreover, most of these studies were conducted in advanced state diabetic patients, with scarce data on pre-diabetes.

For the management of HIV patients who develop diabetes, the added responsibilities can be overwhelming. The intersection among these two chronic diseases might compromise the treatment regimens required for both conditions. Thus, prudent choices for cART must be done for diabetic patients or those at higher risks to develop it.

In fact, antiretroviral represent a very dynamic group in terms of drug innovation, with different mechanisms of action and a considerable number of new drugs that became available in the last 20 years, increasing the possibility of perform a rational and personalized prescription. To give new insights on this context, the effects of co-pathologies in a particular antiretroviral pharmacokinetics and the consequent effects on its antiretroviral response as well as its toxic effects are worth to pursue.

A good number of reported data is on PIs class of antiretrovirals (Riddle *et al.*, 2002; Noor *et al.*, 2004; Schutt *et al.*, 2004). Currently, it is suggested the avoidance of PI-based regiments in patients with pre-existing glucose abnormalities as well as the switch the PI to another drug in patients who developed diabetes related cART. The use of the old NRTI drugs was also associated to a higher risk for diabetes (Gerschenson and Brinkman, 2004; Capeau *et al.*, 2012). In respect to second-generation antiretroviral, there is few reported information, once enough time has not been passed yet, to chronic effects associated with this drugs to be evaluated. While NVP is available since 1996 and is a first generation NNRTI it stands out positively in this scenario. There are not many data on NVP in the context of pre-diabetes/diabetes but this antiretroviral has been described in general as a glucose friendly drug (Eastone and Decker, 1997; De Wit *et al.*, 2008), and in particular has a protective factor for pre-diabetes in HIV-population, by improvement of insulin sensitivity and fasting glycemia (Blanco, 2004; Srivanich *et al.*, 2010). In fact, the mechanism underlying to this good influence has not yet been found.

In some HIV-infected patients who developed glucose disorder during treatment, the switch of the antiretroviral could implicate a reduction of therapeutic benefits and sometimes the better option prescribe an anti-diabetic drug with possibility of a drug-to-drug interactions to occur (Fichtenbaum and Gerber, 2002). However, some of the medications used to treat diabetes and the antiretroviral medication have similar metabolic pathways. Moreover, antiretroviral agents can influence the activity of the CYP P450 system as well as the activity other intervenient on drug metabolism (Xu *et al.*, 2005). This is the case of NVP, a well-known CYP3A4 inducer (Pereira, 2012a). This interaction may change the efficacy and increase the risk of toxic effects of the both drugs.

On the other hand, the influence of glucose abnormalities and insulin resistance in pharmacokinetic and pharmacodynamic of several drugs has been consistently recognized (Dostalek *et al.*, 2012). Although, on the regard of antiretroviral drugs scarce information exists. Antiretroviral are drugs with a narrow therapeutic range for each a variation in PK might produce an increased incidence of sub-therapeutic concentrations and the consequent virologic failure or increased plasma concentrations and higher risk of adverse effects (Liu *et al.*, 2010).

**The choice of the animal model and the related experimental conditions**

In the light of the study of pre-diabetes condition influence on NVP PK, female *Wistar* rats on a sucrose diet were used as study model. Female rats were used due to sex-dependent dimorphic profile of the adverse reactions of NVP, with woman being at an increased risk (Ho *et al.*, 1998; Bersoff-Matcha *et al.*, 2001). Female sex has been shown to be a risk factor for clinically relevant adverse drug reactions (Anderson, 2005; Soldin *et al.*, 2011) and a pharmacokinetic variation has been implicated as the main factor underlying the increased rate and wider range of drug-induced toxicity reactions in women (Miller, 2001; Anderson, 2008). Regarding NVP, a dissimilar phase I metabolite profile was found among men and women: a higher plasma level of the metabolites 12-OH and 3-OH NVP in female sex (Marinho *et al*., 2013). Moreover, females have higher CYP 3A4, 2A6 and 2B6 activities (Anderson, 2008; Scandlyn *et al.*, 2008). These enzymes are involved in NVP phase I biotransformation. About phase II reactions, female mice showed higher hepatic mRNA levels of SULT1A1 (isoenzyme involved in the 12-OH NVP bioactivation) compared with male mice (Alnouti and Klaassen, 2006; Suzuki *et al.*, 2012). On the other hand, women have lower UDP-glucuronosyltransferase activity which suggests that may be prone to less efficient detoxification of phase I metabolites than men (Anderson, 2008; Gallagher *et al.*, 2010).

Species differences on drug metabolism are well known (LeCluyse, 2001; Martignoni *et al.*, 2006). This turns the choice of the animal model a challenging task. For instance, human liver contains less cytochrome P450 per gram than the liver of rat and a faster rate of drug biotransformation and elimination in rats (10 a 20 times faster than in man) (Martignoni *et al.*, 2006)). Since it is intended to mimic in rat the humans effect, it was necessary a higher dose of the drug, equivalent to human therapeutic dose. Regarding NVP, Riska *et al.* (1999) demonstrated that rat specie is a good toxicological, metabolic and pharmacokinetic model for the study of NVP (Riska *et al.*, 1999b). The

phase I metabolite profile is similar among rat and man with the proportion of metabolites being similar and 12-OH NVP being the major phase I metabolite (Riska *et al*, 1999b). Adaramoye *et al.* used 18 mg/kg body weight NVP dose (equivalent of human therapeutic dose, 400 mg of NVP are taken once a day) by oral gavage in a study on the toxicological effect of nevirapine on the liver, kidney and testis of male *Wistar* rat (Adaramoye *et al.*, 2012). As, in other ongoing work of the team, the carcinogenic/mutagenic potential (Antunes *et al.*, 2008) of NVP is being explored, to be surely able to measure NVP phase I metabolites and as a first pilot study with only 7 days of NVP exposition, a toxic dose of NVP 40 mg/kg/day was chose.

As NVP intake is by an oral administration (Boehringer Ingelheim Pharmaceuticals, 2013) and as it was intended to study PK variations, oral administration was selected as the administration route. However, the drug administration by gavage might induce stress in animals and since increased sympathetic activity is associated with the development and worsen of diabetes (Landsberg, 2006). Therefore this way of administration was avoided.

The drug dissolution is one of the main factors to determine its absorption (Mitchell *et al.*, 2003). As methylcellulose (MC) enhances drug dissolution properties, NVP was solved in MC 0.5% w/v since it is practically insoluble in water (Mitchell *et al.*, 2003; Srivastava *et al.*, 2010). In the preliminary experiments, it was found that the volume of drinking water intake per day for each animal was equal to the one observed when NVP-MC was included in drinking water. Each rat drank approximately 30 mL of water per day. Thus, NVP was administered orally in drinking water of the animals.

In the present work the pre-diabetic model chose was the high sucrose diet (HSu) model. When HSu diet is administered for few weeks (more than 4 and less than 9) a lean model of insulin resistance and hypertention is obtained (Ribeiro *et al.*, 2005; Conde *et al.*, 2012). Currently, there are several animal models of type II diabetes, for example *Zucker fa/fa* rat which have a spontaneous recessive mutation in the *fa* gene and the fat-fed/streptozotocin (STZ) model (Wang *et al.*, 2007). This last model is an obese model of insulin resistance and hypertension and is achieved by submitting the animals to a high fat diet. However, although high fat diet induce insulin resistance, is incapable of inducing hypoinsulinemia at least until 12 weeks of administration (Reed *et al.*, 2000). Therefore, hypoinsulinemia is achieved by submitting the high fat animals to a low dose of STZ, inducing necrosis of beta-cells and diminishing diminish the secretion of insulin (Reed *et al.*, 2000). However, obesity is not a preponderant characteristic in HIV-population (Amorosa *et al.*, 2005).

**Is there a change in NVP pharmacokinetics due to pre-diabetes?**

The level of NVP was lower in both liver and plasma, suggesting that NVP is being less absorbed or highly biotransformated and eliminated. Moreover, the 12-OH NVP levels are lower in both liver and plasma, but 2-OH NVP levels do not vary.

Drug absorption is decreased in patients with diabetes (Zhu, 1993; Nowak *et al.*, 1995; Jung *et al.*, 2003; Wu *et al.*, 2004). A plausible mechanism is an increased first past effect in diabetic condition. Little is known about the transporters that influence the disposition of NVP; however it was recently discovered a role for the multidrug resistance protein 7 (MRP7) and multidrug resistance type I (MDR1) encoded by the adenosine triphosphate-binding cassette gene ABCC10 and ABCB1, respectively, in the efflux transport of NVP (Ciccacci et al, 2010; Liptrott *et al.*, 2012). While the effects of diabetes in these transporters are poorly characterized, its activity has not changed in hyperglycemic pregnant woman on insulin (Anger *et al*, 2012).

At first glance it would be said that the lower levels of 12-OH NVP in both liver and plasma were expected in pre-diabetes as NVP is being less bioavailable in these animals. However an interesting result was observed: the ratios NVP/2-OH and NVP/12-OH were inferior in liver of pre-diabetic rats, which revealed a higher biotransformation of NVP into its phase I metabolites. This higher biotransformation may explain the low disposition of NVP found in plasma and liver. Moreover, this effect was more pronounced for 2-OH NVP. While, different CYP isoenzymes are involved in 2-OH NVP (CYP3A) and 12-OH NVP formation (CYP2A6, 2D, 2C9 and 2C19), its functionality seems not be diminished in pre-diabetic animals treated with NVP. Moreover, NVP is a well know CYP inducer (Pereira 2012a) and this increased conversion into 12-OH NVP and 2-OH NVP suggests that this inductor effect is not lost in diabetes. The effect of diabetes in CYP is dependent on the particular CYP isoenzyme, disease status and the model used for the study (Kotlyar and Carson, 1999; Borbas *et al.*, 2006, Dostalek *et al*, 2011; Brill *et al*, 2012).

The pre-diabetic rats showed an increased formation of 2-OH NVP (evaluated by the metabolite rate) and higher levels of this metabolite.

But, surprisingly, pre-diabetic rats showed an increased formation of 12-OH NVP and lower levels of this metabolite. As β-glucuronidase appears be increased in diabetes (Perdichizzi *et al.*, 1983), a plausible explanation for reduced NVP absorption is that an increased first pass effect due to an efficient phase II reactions and drug excretion. Moreover, it might also explain while the 12-OH NVP formation is higher, while plasmatic levels are lower. The 2-OH NVP behaved different. Data on UGT enzymes involved in the biotransformation of each particular metabolite are scarce to establish the comparison.

Remarkably, is via phase II sulfotransferases (SULT) that 12-OH NVP is bioactivated to electrophilic metabolites, which represents the first steps of the mechanism of its hepatotoxicity. Moreover, the electrophiles effects are minimized by glutathione which levels are diminished in diabetes (Dincer *et al.*, 2002; Sekhar *et al.*, 2011). In order to explore this issue, several experiments

as SULT expression experiments, phase II metabolites quantification, markers of hepatotoxicity (liver function tests) must be conducted.

Summing up, besides decreasing NVP absorption, pre-diabetic condition changes the metabolite pattern of NVP: the 2-OH NVP was the major metabolite formed instead of 12-OH NVP. This can have interesting implications on NVP toxicity, as 12-OH NVP has been described as the main player metabolite on NVP-associated toxic events (Caixas et al, 2012, Pereira et al, 2012). Moreover, while the lower NVP absorption, also decreases the risk of its toxicity (de Maat *et al.*, 2003; Almond *et al.*, 2004; Dailly *et al.*, 2004; Kappelhoff *et al.*, 2005; Hall and MacGregor, 2007; Stohr *et al.*, 2008; Wyen *et al.*, 2008; Dong *et al.*, 2012; Ratanasuwan *et al.*, 2012) this can have strong implications on drug efficacy. NVP is a drug with a narrow therapeutic window and a low genetic barrier (AIDS, 2012) and its sub-exposition might lead to resistance development and virological failure with serious therapeutic implications in these patients.

It is concluded that NVP PK is strongly influenced by HSu diet with plausible diminishing of its absorption and a different metabolite profile pattern. The finding of a decreased absorption can have significant impact on the NVP dose to be used in IR patients. Moreover on basis of the present data it is not possible to describe the fate of 12-OH NVP metabolite and the consequent implications on NVP toxicity. Also, further experiments should explore the increased formation of 2-OH NVP.

**NVP and pre-diabetes: friend or foe?**

Pre-diabetic animals treated with NVP showed no variation in arterial pressure, a benefic decrease in fasting glycemia and no influence in insulin sensitivity. Pre-diabetic patients have a proneness to present a superior mean blood pressure comparatively with healthy populations (Ryden *et al*., 2007). Also, long term cART use has been associated with an increase risk for heart disease (Dube *et al.,* 2008; Gopal *et al*., 2009), but the regiments based on non-nucleoside reverse transcriptase inhibitors (NNRTI) seem to be more heart friendly (van Leth *et al.,* 2004b; Parienti *et al.,* 2007; Maggi *et al*., 2011). Moreover, several studies reported that NVP raises HDL levels, resulting in an improvement of the atherogenic index of patients (van der Valk *et al*., 2001; van Leth *et al*., 2004a). 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*., 2004a; Sankatsing *et al*., 2007; Franssen *et al*., 2009). Moreover, it was recently indicated as a protective factor in pre-diabetes (Srivanich et al., 2010). On this regard NVP might be a good choice for patients with multiple cardiovascular risk factors as hypertension, dyslipidemia or diabetes.

The treatment with NVP improves fasting glycemia in insulin resistant group. This fact is in agreement with the clinical studies reporting that NVP might be a protective factor for prediabetes (Srivanich et al., 2010). Moreover, Blanco and Shahmanesh observed that subjects treated with NVP had significantly lower glucose in plasma than those on protease inhibitor or on the other first generation NNRTI-efavirenz (Blanco, 2004; Shahmanesh *et al*., 2004). The preliminary data, suggest

that the lower NVP absorption and the lower availability of 12-OH NVP found in diabetic condition might be associated to less liver toxicity and thus improving fasting glycemia. In addition, while no liver function tests were performed, a toxic concentration of NVP was administered and even though this benefic effect is found.

According to the previous results about glycemia, would be expected an increase of insulin sensitivity (increase KITT), since the lower glucose observed is the result of the normal detection by β-cells in the pancreas, which leads to the release of insulin. The binding of insulin to the cell membrane (muscle, fat or brain cell) sets off a cascade of molecular events that allows glucose uptake. A decreased production of glucose instead an increased uptake by liver, could explain the low glucose level found and thus the impaired insulin sensitivity. Further studies might be performed to explore the mechanisms underlying the improvement of glucose as the pyruvate dehydrogenase kinase-4 activity or GLUT4 expression (Jeoung and Harris, 2008). Other variables as a prolonged exposition time to NVP, different NVP doses and different IR models are important to explore.

The majority of the antiretroviral drugs impair glucose homeostasis and insulin resistance (Mulligan et al., 2000; Murata et al., 2000; Justman et al., 2003; Noor et al., 2004;Brown et al., 2005; Tien et al., 2007; De Wit et al., 2008; Stanley et al., 2009). For instant, different mechanisms have been implied to this issue as mitochondrial dysfunction associated to NRTI and inhibition of glucose uptake into adipocytes via the GLUT4 glucose transporter for PI. For NVP, almost no information exists. Hong-Brown et al. observed a decline in phosphorylation of protein 4EBP1 in C2C12 myocytes associated to NVP treatment (Hong-Brown et al., 2005). This protein is involved in insulin signaling (Gingras et al., 1998; Wang et al., 2006). So, this represents a good starting for further studies.

**Do pre-diabetes predispose to higher NVP toxicity?**

The pre-diabetic animals presented lower NVP and lower 12-OH NVP exposition, which raises the hypothesis of lower toxicity in this context.

Recent evidence on the mechanism of NVP toxicity, point towards the bioactivation of the 12-OH NVP metabolite into reactive electrophile 12-sulfoxy NVP which has the ability to covalently modify proteins and incite the pathway for liver injury progression (Antunes *et al.,* 2010a; Antunes et al., 2010b; Caixas *et al.,* 2012; Pereira *et al*., 2012a). This liver injury progression will preferentially occur after glutathione depletion. Low glutathione level is characteristic of pre-diabetes (Dincer *et al*., 2002; Sekhar et al., 2011). As so, total aminothiols levels were evaluated in order to explore the effect of NVP on endogenous antioxidant defense in pre-diabetes. The non-treated pre-diabetic animals presented the great variations in total aminothiols content in plasma and liver.

Aminothiols are low molecular weight thiol-containing aminoacids that provide numerous roles in metabolism and homeostasis, particularly as redox buffers and as components in antioxidant defense (Nolin et al., 2007). The impairment of HCys, Cys, GSH and Cys-Gly redox thiol status might be linked to the occurrence of several disorders. All these species are interrelated and present a responsibility in

redox environment and free radical interactions, by acting as redox buffers (Giustarini et al., 2006). In the presence of reactive oxygen species (ROS), they form disulfides in plasma to remove the danger compound. Moreover, they also detoxify electrophilic endogenous species as well as the ones formed upon drug bioactivation (Antunes et al., 2010a). However, the production of the free radical and the electrophilic moieties may exceed the antioxidant capacity of the system (oxidative stress), which favors the damage of proteins, lipids and acid nucleic (Dalle-Donne et al., 2006). Oxidative stress is considered to be the main factor in the development of diabetic complications and tissue injury (Baynes, 1991; Maritim et al., 2003). Non-enzymatic glycation, metabolic stress secondary to the changes in energy metabolism and tissue injury resulting from the changes in antioxidant defense system are some mechanisms involved in the increase of oxidative stress in diabetes (Baynes, 1991). In this work, the total aminothiols were measured which include the free forms (reduced and oxidized) and protein-bound forms of all aminothiols. However, the free forms may be more likely to play a role in the pathogenesis of diabetes.

The normal level of total plasma HCys, which includes free HCys, homocystine (HCysSS), and mixed disulfides is between 5 and 15 μM (Kaul et al., 2006; Antoniades et al., 2009). The levels of tHCys observed are in this range. In diabetes, it is described a higher plasmatic levels of homocysteine (de Luis et al., 2005) as it was obtained in the present work. In fact, both hyperglycemia and hyperhomocysteinemia lead to a diabetic cardiomyopathy, which is the leading cause of morbidity and mortality in this population (Mishra et al., 2010). Across the groups there were no differences of HCys in plasma and liver.

Cys is the aminothiol most abundant in plasma which is in agreement with literature (Dickinson and Forman, 2002; Go and Jones, 2011). Interestingly, Cys is the precursor for 3-phophoadenosine 5-phosphosulfate (PAPS) (Baker, 2006) which serves as the universal sulfonate donor for all sulfotransferase reaction (Venkatachalam, 2003). This might suggest that no higher requirement of Cys and consequently PAPS is necessary in IR. Along with this hypothesis, IR group might not be at increased risk of 12-OH NVP bioactivation. Interestingly, it was recently found a decreased in sulfotransferase activity in diabetes (Yalcin et al., 2013). Although, an important safeguard for this data interpretation is that scarce information exist on PAPS levels and SULT activities in our model.

Glutathione is the most important intracellular thiol antioxidant; is crucial for maintaining redox homeostasis, protecting cell from oxidative stress. Liver has one of the highest organ contents of GSH (Dickinson and Forman, 2002; Go and Jones, 2011). It is reported that the diminished GSH level verified in diabetic patients is attributed to limited precursor availability, namely Cys (Sekhar et al., 2011). So, GSH level depends on Cys disposition and with a dietary supplementation with Cys the GSH level can improve and might be a good option to diabetic patients. In the present study, total GSH content was negatively influenced by pre-diabetes, although no effect on Cys was observed. Moreover, NVP does not influence total GSH and total Cys or any other thiol with the exception of Cys-Gly.

Cys-Gly is an aminothiol that derive for the breakdown of glutathione (Pastore et al., 1998). So, the higher is glutathione, the lower is Cys-Gly and vice versa. As Cys-Gly is one of the products of extracellular GSH hydrolysis, there is a higher level in plasma than in liver. The higher Cys-Cly level in

IR plasma and the lower level in Cys-Gly IR-NVP group are also in agreement that in HSu animals, NVP biotransformation suffers a shift towers 2-OH NVP pathway and a decrease in the risk of liver injury.

# 5. Conclusion

**Is NVP a good option for pre-diabetic HIV-infected patients?**

The data presented in this study revealed a clear influence of pre-diabetes in NVP pharmacokinetic. On the on hand, it seems have a negative influence since the decreased bioavailability of NVP in pre-diabetes might suggest an adjustment of NVP dose in pre-diabetic HIV-infected patients. On the other, it appears be a positive influence since the lower absolute concentration of metabolites and the higher phase I biotransformation in pre-diabetic rats suggest an increase of glucuronoconjugation which provide the metabolites excretion and eliminates the chance of the formation of reactive electrophiles (12-sulfoxy NVP). This hypothesis was also proved by the aminothiols results. In pre-diabetic rats was observed a slightly increase of antioxidant defenses after NVP treatment. Moreover, the similar level of Cys (crucial for sulfotransferase activity) observed among groups suggest that pre-diabetes seems not predispose to a higher 12-sulfoxy formation.

In respect of NVP role, it might be a good option for HIV-infected patients at higher risk of develop pre-diabetic condition since the results revealed an improving of fasting glycemia.

In this study were observed interesting results which could, in the future, provide a better care to HIV-infected patients with pre-diabetes. However, further studies must be conducted in order to understand the mechanisms underlying these changes. Also, it would be interesting to do a similar study in HIV infected patients with pre-diabetes. In that case, the influence of HIV infection in diabetes panorama would be investigated, which was not possible to do in this study.

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# 7. Annex

## Annex 1

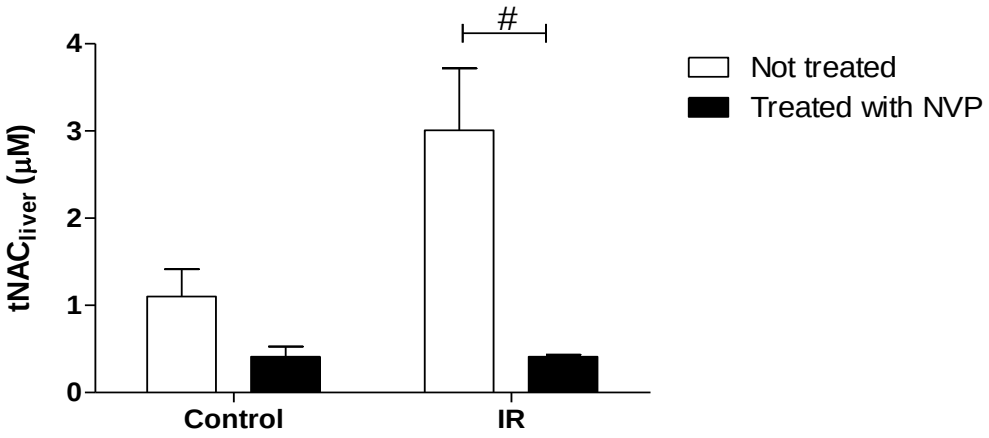


**Figure 7.1 - Liver concentrations of tNAC**
(□) Not treated (*n* = 4 *per* group); (■) Treated (*n* = 4 *per* Control and *n* = 2 *per* IR). Concentration of tNAC (mean ± SEM) is expressed in µM. Data are statistically different between control and insulin resistant groups (C and IR) $^{\#}P<0.05$ (*Two-way ANOVA* with ***Bonferroni's*** Multiple Comparison test).