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Differences in nevirapine biotransformation as a factor for its sex-dependent dimorphic profile of adverse drug reactions

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Objectives: Nevirapine is widely used for the treatment of HIV-1 infection; however, its chronic use has been associated with severe liver and skin toxicity. Women are at increased risk for these toxic events, but the reasons for the sex-related differences are unclear. Disparities in the biotransformation of nevirapine and the generation of toxic metabolites between men and women might be the underlying cause. The present work aimed to explore sex differences in nevirapine biotransformation as a potential factor in nevirapine-induced toxicity.

Methods: All included subjects were adults who had been receiving 400 mg of nevirapine once daily for at least 1 month. Blood samples were collected and the levels of nevirapine and its phase I metabolites were quantified by HPLC. Anthropometric and clinical data, and nevirapine metabolite profiles, were assessed for sex-related differences.

Results: A total of 52 patients were included (63% were men). Body weight was lower in women ($P=0.028$) and female sex was associated with higher alkaline phosphatase ($P=0.036$) and lactate dehydrogenase ($P=0.037$) levels. The plasma concentrations of nevirapine ($P=0.030$) and the metabolite 3-hydroxy-nevirapine ($P=0.035$), as well as the proportions of the metabolites 12-hydroxy-nevirapine ($P=0.037$) and 3-hydroxy-nevirapine ($P=0.001$), were higher in women, when adjusted for body weight.

Conclusions: There was a sex-dependent variation in nevirapine biotransformation, particularly in the generation of the 12-hydroxy-nevirapine and 3-hydroxy-nevirapine metabolites. These data are consistent with the sex-dependent formation of toxic reactive metabolites, which may contribute to the sex-dependent dimorphic profile of nevirapine toxicity.

Keywords: nevirapine toxicity, nevirapine metabolism, sex differences, sex-dependent pharmacokinetics

Introduction

The availability of combined antiretroviral therapy (cART) has changed the prognosis of HIV infection in properly medicated patients from a lethal disease into a chronic condition. However, HIV-positive individuals still face obstacles associated with chronic cART use, particularly related to long-term adverse events.[1]

Nevirapine was the first approved non-nucleoside reverse transcriptase inhibitor (NNRTI),[2] and is still the most prescribed drug among this class. It is also the most used antiretroviral in countries with limited economic resources, particularly due to its low cost.[3] One of the most relevant benefits of nevirapine is its efficacy in the prevention of mother-to-child HIV transmission, with the drug being commonly indicated for pregnant women and their children.[3–6]

Despite its clinical efficacy, nevirapine is associated with hepatotoxicity and skin rash. These reactions may lead to drug discontinuation or even be fatal.[5,7–9] Moreover, nevirapine can induce hepatocellular neoplasias in animal models,[10] 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.[1]

Although the mechanisms underlying nevirapine toxicity are still not fully understood, the current evidence is strongly consistent with a higher risk in women[11–13] and a subjacent immune mediation.[9,14–16] To allow for sex differences in immune hyper-reactivity,

it is recommended that in cART-naive women nevirapine 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.[17]

The attempts to correlate nevirapine exposure and toxicity outcomes have so far been inconclusive,[18–22] which suggests that nevirapine biotransformation to reactive metabolites, rather than the levels of the parent drug, may be the basis of the drug's adverse effects. In humans, nevirapine biotransformation involves the generation of several hydroxylated phase I metabolites (at nevirapine positions 2, 3, 8 and 12; Figure 1).[23,24] 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[25,26] capable of binding covalently to proteins and other biomacromolecules (Figure 1).[25–32] The involvement of phase II reactions, and particularly the sulphotransferase (SULT)-mediated formation of 12-sulphoxy-nevirapine from 12-hydroxy-nevirapine, has recently gained increased support as a major player in this context,[25,27,29–32] with protein adducts from this pathway having already been detected in patients on therapeutic doses of nevirapine.[28,29]

These new insights into the implications of nevirapine biotransformation highlight the importance of exploring sex-related variations in the formation of toxic nevirapine metabolites, which might explain the higher risk for nevirapine toxicity in women. The aim of the current work was to investigate sex differences in phase I nevirapine biotransformation in HIV-infected patients.

Patients and methods

Patient inclusion, data gathering and blood sampling

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 Committee for Data Protection (process number 6567/2009). The patients gave their written informed consent and adherence was controlled by the clinician. All patients were adults with documented HIV infection who had been using nevirapine-containing cART (400 mg once daily) for at least 1 month, regardless of the past therapeutic history. Exclusion criteria were being <18 years of age, having AIDS-defining conditions, and compliance issues. The following

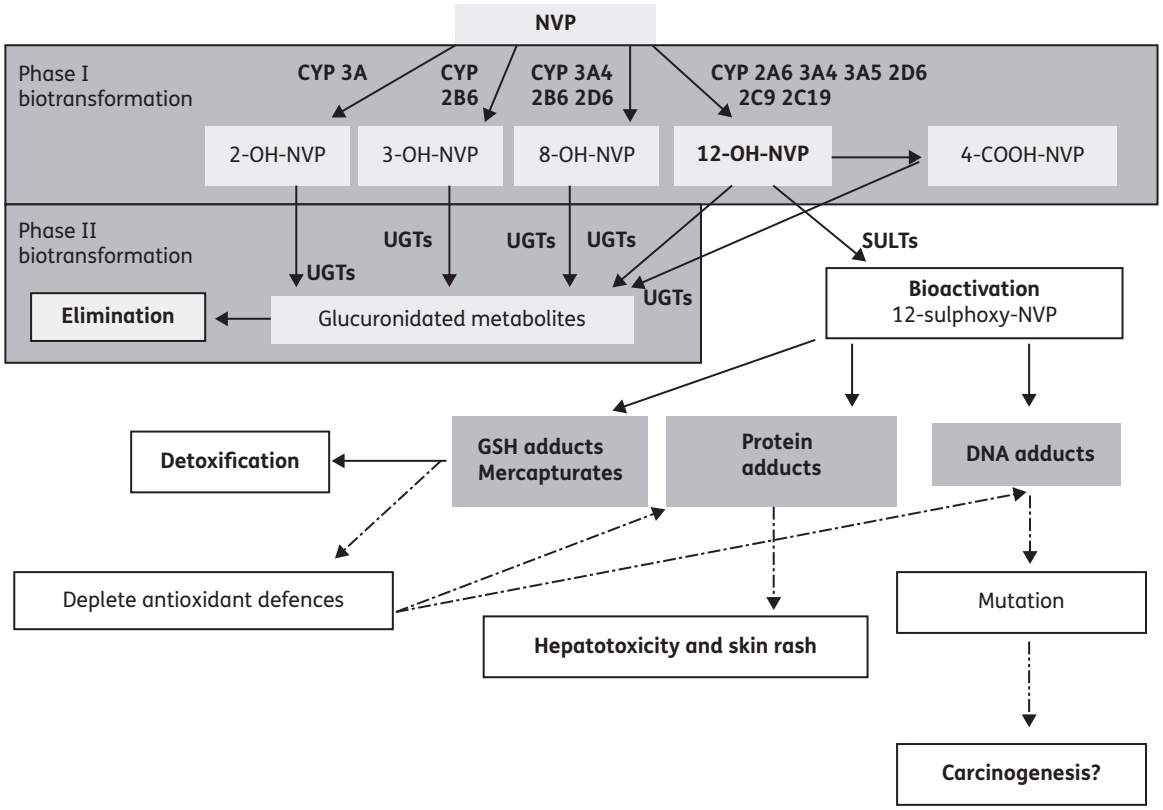

Figure 1. Nevirapine biotransformation, disposition and proposed bioactivation pathways. Nevirapine (NVP) is metabolized by several isoforms of cytochrome P450 (CYP), yielding several phase I metabolites: 2-hydroxy-nevirapine (2-OH-NVP), 3-hydroxy-nevirapine (3-OH-NVP), 8-hydroxy-nevirapine (8-OH-NVP) and 12-hydroxy-nevirapine (12-OH-NVP).[24] 12-OH-NVP is further oxidized by CYP450 to yield 4-carboxy-nevirapine (4-COOH-NVP).[25] The phase I nevirapine metabolites undergo extensive glucuronidation via UDP-glucuronosyltransferase (UGT), which represents a major pathway of nevirapine elimination.[24] The bioactivation of 12-OH-NVP by SULTs can generate 12-sulphoxy-nevirapine (12-sulphoxy-NVP), a reactive metabolite that binds covalently to proteins and DNA.[28,29,53] The formation of DNA adducts could explain the increased incidence of non-AIDS-defining cancers among HIV-infected patients treated with NNRTIs.[1] Also, the formation of adducts with proteins could explain the nevirapine-associated adverse reactions, hepatotoxicity and skin rash.[15] The presence of glutathione (GSH) adducts and mercapturates in patients and animal models treated with nevirapine has also been detected.[26]

data were gathered for each patient: age, ethnicity, weight, height, time on nevirapine, time between last nevirapine intake and blood sampling, viral load, CD4+ cell count and hepatic function biomarkers [alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALKP) and bilirubin]. Blood samples (2 mL) were collected into EDTA-containing tubes.

Extraction of analytes from blood samples and HPLC quantification

Plasma was obtained by centrifugation of blood at 3000 **g** for 10 min. Aliquots of the plasma (900 μL) were heated at 60°C for 60 min for viral inactivation before handling at room temperature.

Pirenzepine was purchased from Sigma–Aldrich (Madrid, Spain) and used as an internal standard. Analytes were then extracted from plasma with dichloromethane (VWR, Radnor, PA, USA).

Nevirapine was obtained from Cipla (Maharashtra, India) and the 2-hydroxy, 3-hydroxy-, 8-hydroxy- and 12-hydroxy-nevirapine metbolites were synthesized as described previously[33,34] and used as standards for HPLC quantification.

HPLC analyses of the nevirapine and metabolite profile were conducted on an Agilent 1100 Series system (Agilent Technologies, Santa Clara, CA, USA) using a reversed-phase Luna C18 (2) column (250 mm×4.6 mm; 5 μm; Phenomenex, Torrance, CA, USA). The mobile phase—10% acetonitrile (VWR) in 15 mM ammonium acetate (Merck, Darmstadt, Germany) 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°C, the injection volume was 100 μL and UV absorbance was monitored at 254 nm. The lower limit of quantification of the method was 10 ng/mL for each analyte.

Statistical analyses

Systemic exposure to the different nevirapine metabolites was assessed in terms of absolute concentrations and as the proportion of each metabolite in the plasma. Mean (±SEM), median (IQR), number and percentage were used to describe the study population. Student's *t*-test was performed for comparisons between means and the Mann–Whitney *U*-test was performed for comparisons between medians. The statistical analyses were performed using Graph Prism® 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

A total of 52 patients (63% men) were included in the study. The anthropometric and clinical data are presented in Table 1.

Body weight was significantly higher in men compared with women, but the body mass index was similar in the two groups. Among the liver function tests analysed, differences were found for LDH and ALKP, which were higher in women.

There were no differences between sexes regarding age, time on nevirapine, time between sampling and last nevirapine intake, CD4 cell counts, ALT, GGT and direct bilirubin; the total bilirubin concentration was higher in men (Table 1). All patients had an undetectable viral load.

Sex differences in the plasma concentration of nevirapine and in its metabolite profile are presented in Table 2. Women had higher nevirapine and 3-hydroxy-nevirapine plasma concentrations, when adjusted for body weight. All patients had 8-hydroxy-nevirapine levels below the lower limit of quantification of our method.

Sex differences in the proportions of nevirapine metabolites are presented in Table 3. Women had a significantly higher proportion of 12-hydroxy-nevirapine and 3-hydroxy-nevirapine compared with men, when the data were adjusted for body weight.

Discussion

Nevirapine is a remarkable example of a drug with a sexually dimorphic profile of adverse drug reactions, with women being at greater risk of experiencing skin and liver toxicity. It has recently been hypothesized that nevirapine biotransformation plays an important role in the onset of these adverse effects.[35] However, sex-dependent differences in nevirapine pharmacokinetics have been

Table 1. Anthropometric and clinical data

Parameter	Men	Women	*P* value
Number of patients	33	19	
Percentage of non-Caucasians	42	32	
Age (years)[a]	50 (39–60)	46 (38–63)	NS
Body weight (kg)[b]	73±2	65±3	0.028
Body mass index (kg/m^2)[b]	25±1	25±1	NS
Time on nevirapine (years)[a]	4 (2–9)	4 (2–7)	NS
Time between sampling and last nevirapine intake (h)[a]	12 (6–15)	13 (11–15)	NS
CD4+ cell count (cells/mm^3)[a]	515 (386–675)	575 (413–735)	NS
ALT (U/L)[a]	36 (23–47)	31 (26–45)	NS
ALKP (U/L)[a]	80 (69–106)	110 (82–155)	0.036
LDH (U/L)[a]	181 (167–243)	228 (196–244)	0.037
GGT (U/L)[a]	70 (36–134)	59 (51–126)	NS
Total bilirubin (mg/dL)[a,c]	0.39 (0.33–0.47) (n=25)	0.33 (0.27–0.40) (n=15)	0.026
Direct bilirubin (mg/dL)[a,c]	0.1 (0.08–0.11) (n=17)	0.1 (0.07–0.11) (n=13)	NS

NS, not significant.
Differences were considered significant if $P < 0.05$.
[a]Mann–Whitney *U*-test, median (IQR).
[b]Student's *t*-test, mean ± SEM.
[c]Missing values correspond to patients for whom clinical data were not available.

Table 2. Plasma concentrations of nevirapine and its phase I metabolites

	Men ($n=33$)	Women ($n=19$)	*P* value
Analyte (ng/mL)			
NVP[a]	4279 (2678–5105)	4233 (3533–5130)	NS
2-OH-NVP[a,b]	58.6 (29.5–109.6) ($n=19$)	63.2 (31.3–80.5) ($n=7$)	NS
3-OH-NVP[a,b]	24.0 (17.2–31.3) ($n=31$)	33.7 (22.7–38.6) ($n=17$)	0.047
12-OH-NVP[a]	364.9 (247.3–543.4)	371.0 (270.2–450.7)	NS
Analyte (ng/mL/kg)			
NVP[a]	53.9 (34.6–72.9)	70.7 (51.8–86.4)	0.030
2-OH-NVP[a,b]	0.73 (0.42–1.78) ($n=19$)	0.92 (0.34–1.73) ($n=7$)	NS
3-OH-NVP[a,b]	0.34 (0.23–0.47) ($n=31$)	0.52 (0.32–0.73) ($n=17$)	0.035
12-OH-NVP[a]	4.80 (3.48–8.47)	6.06 (3.87–7.79)	NS

NS, not significant; NVP, nevirapine; 2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine.
Differences were considered significant if $P<0.05$.
[a]Mann–Whitney *U*-test, median (IQR).
[b]Missing values correspond to patients for whom the metabolite concentration was below the lower limit of quantification for the method.

Table 3. Sex differences in the proportions of the major nevirapine phase I metabolites

	Men ($n=33$)	Women ($n=19$)	*P* value
%			
2-OH-NVP[a]	11.1 (8.2–18.5)	9.6 (7.8–21.9)	NS
3-OH-NVP[b]	5.5 ± 0.4	7.5 ± 0.8	0.013
12-OH-NVP[a]	88.2 (79.8–94.7)	90.8 (83.2–93.8)	NS
%/kg			
2-OH-NVP[a]	0.17 (0.11–0.26)	0.15 (0.08–0.42)	NS
3-OH-NVP[b]	0.08 ± 0.01	0.12 ± 0.02	0.001
12-OH-NVP[a]	1.24 (1.04–1.29)	1.35 (1.17–1.76)	0.037

NS, not significant; 2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine.
Differences were considered significant if $P<0.05$.
[a]Mann–Whitney *U*-test, median (IQR).
[b]Student's *t*-test, mean ± SEM.

poorly explored. In the present work, sex differences in the biotransformation profile of nevirapine were studied in order to explore their potential role in nevirapine toxicity. We found that the nevirapine phase I metabolite profile was dissimilar between men and women, with differences being most noteworthy for the 12-hydroxy-nevirapine and 3-hydroxy-nevirapine metabolites, which had higher plasma levels in women.

Pharmacokinetic variation has been implicated as the main factor underlying the increased rate and wider range of drug-induced toxicity reactions in women.[36,37] These effects have often been purported to be related to higher drug bioavailability in females.[36–40] These pharmacokinetic differences may arise due to variations in endogenous and exogenous hormones, and also in liver metabolism.[36] Moreover, body size and fat composition are also thought to contribute. Females typically have a lower body weight and size than males, and also a higher percentage of body fat, which might influence the distribution volume of drugs, in particular those that are highly lipophilic, such as nevirapine.[37]

In fact, a relationship between lower body weight, lower nevirapine clearance and higher nevirapine toxicity has been described.[41–43] However, the attempts to demonstrate that patients experiencing higher plasma nevirapine levels are at greater risk for nevirapine toxicity have failed, as divergent results have been obtained.[16,18,20–22,44–47] Nonetheless, it is important to highlight that body weight adjustment was rarely performed in these studies.[20–22,44,45]

As expected, in our study population women had lower body weights than men. Additionally, the sex differences found in nevirapine biotransformation were more pronounced when normalization per unit body weight was performed. This excludes lower body weight as the only factor explaining the different nevirapine biotransformation between the sexes. The absence of a correlation between nevirapine concentration and toxicity[20–22,45] might suggest that nevirapine *per se* is not toxic, but can form toxic metabolites upon biotransformation.[48]

Nevirapine is biotransformed into several hydroxylated metabolites via phase I cytochrome P450 mediation (Figure 1). Females have higher CYP3A4, 2A6 and 2B6 activities, while sex differences in CYP2C9 and 2D6 have not been described.[37,49,50] Moreover, evidence obtained from pharmacogenetic data has suggested an influence of CYP2C19 on nevirapine plasma levels,[51] although without specifying the particular metabolite(s) generated. This CYP isoenzyme has also been linked to the generation of reactive metabolites capable of binding to glutathione and forming nevirapine–glutathione adducts *in vitro*.[31] Recently, the involvement of CYP2C19 in the formation of 12-hydroxy-nevirapine was excluded,[52] but its influence in the generation of the other phase I metabolites was not assessed. The reported higher CYP3A4, 2A6 and 2B6 activities in females are consistent with the higher proportions of 12-hydroxy-nevirapine and 3-hydroxy-nevirapine found for women in the current work (Figure 1 and Tables 2 and 3).

Previous work by Hall and MacGregor[22] did not identify any strong correlation between plasma levels of nevirapine or its major phase I metabolites and either hepatotoxicity or skin rash events, or sex differences in metabolite proportions. Nevertheless, no adjustment per unit of body weight was performed in that study. Also, it is important to highlight that the authors performed an extraction of the analytes

preceded by a glucuronidase treatment step. This step precluded an estimation of the levels of free phase I metabolites, which prevents direct comparison with our data.

The formation of glucuronides (Figure 1) is a major route of elimination of nevirapine phase I metabolites.[24] However, the involvement of phase II metabolic pathways, namely sulphonation, cannot be excluded.[48] For instance, the bioactivation of 12-hydroxy-nevirapine by SULTs has been increasingly indicated as the plausible mechanism for nevirapine-associated toxicity.[25–30,32,48]

12-Hydroxy-nevirapine is a non-reactive metabolite *per se*; however, it can be bioactivated by SULTs in the liver and skin, yielding the reactive species 12-sulphoxy-nevirapine.[32] Moreover, using 12-mesyloxy-nevirapine as a synthetic surrogate for 12-sulphoxy-nevirapine, we have shown covalent binding of this reactive electrophile *in vitro* to several amino acids,[27] haemoglobin and human serum albumin,[30] as well as nucleosides and DNA.[53,54] We have also demonstrated for the first time the presence of 12-hydroxy-nevirapine-derived haemoglobin adducts in HIV-infected patients.[28] More recently, Sharma *et al*.[32] showed covalent binding of 12-sulphoxy-nevirapine to skin proteins after incubation of this reactive metabolite with skin homogenate. Likewise, recent work by Meng *et al*.[29] showed evidence for the formation of nevirapine–human serum albumin adducts, consistent with reaction with 12-sulphoxy-nevirapine. Nevertheless, the detection of 12-sulphoxy-nevirapine in man has not yet been achieved.

Recent evidence has shown that recombinant human SULT1A1*1 is capable of converting 12-hydroxy-nevirapine into 12-sulphoxy-nevirapine.[32] Owing to its broad spectrum of substrates and high hepatic and extrahepatic expression, SULT1A1 appears to be the main form of human SULT involved in the detoxification of xenobiotics, particularly phenolic metabolites.[55] Nonetheless, further studies are needed to clarify whether other SULTs play a role in the bioactivation of 12-hydroxy-nevirapine[56] (Figure 1), as well as whether the isoforms involved are the same in skin and liver.

It is known that SULT1A1 is highly polymorphic and that there are marked differences in the activities of SULT1A1 variants (1A1*1, 1A1*2 and 1A1*3).[57] These differences may determine distinct susceptibilities to nevirapine toxicity and also the tissue-specific responses. While it is difficult to explain sex-related differences on the basis of genetic polymorphisms, as there is no evidence for a sex-dependent pattern in the frequencies of polymorphic SULTs, sex-dependent enzymatic regulation of SULTs or differences in 3′-phosphoadenosine 5′-phosphosulphate (PAPS) availability are plausible.

Little is known about the sexually dimorphic expression of SULTs in humans, but sex-divergent SULTs are mostly female predominant in mice;[58] for instance, female mice showed higher hepatic mRNA levels of SULT1A1 compared with male mice.[58–60] Likewise, higher expression of SULT1D1 was reported in the canine female liver.[61] In addition, a female predominance in SULT2A1/2A2 has been reported in mice[59,62] and rats.[63,64] It is also noteworthy that Alnouti and Klaassen[58] demonstrated that androgens and a male pattern of growth hormone secretion can have a suppressive effect on the expression of some SULTs in mouse hepatic tissue, while oestrogens and a female pattern of growth hormone secretion can exert opposite effects. Furthermore, the activity of human SULT2 enzymes, including SULT2B1b, which is expressed in the skin and is capable of sulphonating a number of xenobiotics, has been shown to undergo modulation by several types of nuclear receptors; among these are peroxisome proliferator-activated receptors (PPARs).[65] It has been argued that metabolic interactions between PPARγ or PPARα and oestrogens, oestrogen receptors or oestrogen receptor-related cofactors could explain, at least in part, some sex-specific differences observed in PPAR-based treatments.[66] Whether or not a sexually dimorphic pattern in PPAR expression is an underlying cause of differential nevirapine toxicity remains to be established.

In addition to 12-hydroxy-nevirapine, current evidence suggests that 3-hydroxy-nevirapine might also undergo bioactivation;[26] however, there is no clear evidence so far associating 3-hydroxy-nevirapine or any 3-hydroxy-nevirapine derivative with nevirapine-related toxic reactions. Interestingly, in the present study, sex differences were found only for the plasma levels of 3-hydroxy-nevirapine and 12-hydroxy-nevirapine, which is consistent with the hypothesis of sex-dependent formation of reactive metabolites. Also, the fact that women have lower UDP-glucuronosyltransferase activity[37,67] suggests that they may be prone to less efficient detoxification of these metabolites than men.

Sex-dependent variations in the expression/activity of PAPS synthase enzymes, which catalyse the biosynthesis of the SULT cofactor PAPS, may also contribute to the different toxicity outcomes. PAPS is the universal donor of the sulphonyl moiety that enables the sulpho-conjugation of SULT substrates. At least three PAPS synthase isoforms (PAPSS1, PAPSS2a and PAPSS2b), with different activities and tissue distributions, have been identified in humans.[68] While PAPSS2b is the main isoform in human liver, PAPSS2a is not expressed in this tissue and PAPSS1 is expressed to a lesser extent in the liver compared with several other tissues.[68] A similar tissue distribution of PAPS synthase isoforms has been reported in mice and, interestingly, the hepatic expression of PAPSS2 in mice has been found to be female predominant.[59] Although sex-related differences in the expression of PAPS synthase enzymes do not appear to have been investigated in humans, it is noteworthy that both PAPSS1 and PAPSS2 are highly polymorphic[69,70] and are differentially expressed in various tissues.[71] In particular, PAPSS1 expression is notably high in the skin,[71] where sulphonation of 12-hydroxy-nevirapine has been associated with nevirapine-induced skin rash.[32]

It should also be noted that the role of immune-mediated responses in the onset of nevirapine-related toxic reactions is well recognized, with higher CD4+ cell counts being associated with a higher risk of hepatotoxicity and skin rash.[5,14] The predominance of autoimmune diseases in women, who have stronger humoral and cellular responses than men, is widely documented.[72,73] While this is likely to contribute to the sex-dependent profile of adverse events related to nevirapine treatment, current therapeutic decisions already take some of these effects into account. In fact, the introduction of lower CD4+ cut-off level criteria for the initiation nevirapine therapy in women than in men has led to a decreased incidence of drug-related toxicity.[17] Nonetheless, reports of adverse effects still persist, suggesting that other factors are at play. Thus, in addition to distinct immune responses, sex-related differences in metabolic activation may contribute to the sexually dimorphic profile of adverse events related to nevirapine treatment.

Nevirapine is among an increasing number of drugs found to display sex differences in pharmacokinetics and in adverse events upon biotransformation. Despite significant progress in recent years, there are still large gaps in our knowledge of the effects of sex upon the clinical pharmacokinetic of nevirapine. Although our present work provides evidence for a sex-dependent

dimorphism in nevirapine metabolism, it does not appear that differences in the concentrations of nevirapine and its phase I metabolites *per se* are the basis for the apparently higher risk of idiosyncratic reactions to nevirapine in women. Future research must be conducted to elucidate the role of SULTs in nevirapine metabolism and bioactivation, and on the elicited sex-related differences in susceptibility to the toxicity of the drug, with a view to the personalized, safer prescription of nevirapine.

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Transparency declarations

None to declare.

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