In vivo profiling and distribution of known and novel phase I and phase II metabolites of efavirenz in plasma, urine and cerebro-spinal fluid

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List of abbreviation:

7-hydroxy-efavirenz	7OH-EFV
8-hydroxy-efavirenz	8OH-EFV
Efavirenz	EFV
Glucuronide	gln
Swiss HIV Cohort Study	SHCS
University Hospital of Lausanne	CHUV

ABSTRACT

Efavirenz (EFV) is principally metabolized by CYP2B6 to 8-hydroxy-efavirenz (8OH-EFV) and to a lesser extent by CYP2A6 to 7-hydroxy-efavirenz (7OH-EFV). Most metabolites profile analyses have been so far restricted to 8OH-EFV, 7OH-EFV and EFV-N-glucuronide even though these metabolites represent a minor percentage of EFV metabolites present *in vivo*. We have performed a quantitative phase I and II metabolites profiles analyses by tandem mass spectrometry of plasma, cerebrospinal fluid (CSF) and urine samples in 71 HIV patients under efavirenz, prior to and after enzymatic (glucuronidase and sulfatase) hydrolysis. We have shown that phase II metabolites constitute the major part of the known circulating efavirenz species in humans. The 8OH-EFV-Glucuronide (8-OH-gln), and 8OH-EFV-sulfate (identified for the first time) in humans were found 64- and 7-fold higher than the parent 8OH-EFV, respectively. In genotyped individuals (n=67) for *CYP2B6*, *2A6* and *CYP3A* metabolic pathways, 8OH-EFV/EFV ratios in plasma were an index of CYP2B6 phenotypic activity (p < 0.0001) which was also reflected by phase II metabolites 8OH-EFV-glucuronide/EFV and 8OH-EFV-sulfate/EFV ratios. Neither EFV nor 8OH-EFV, nor any other considered metabolites in plasma were associated with an increased risk of central nervous system (CNS) toxicity. In CSF, 8OH-EFV levels were not influenced by CYP2B6 genotypes and did not predict CNS toxicity. The phase II metabolites 8OH-EFV–gln, 8OH-EFV-sulfate and 7OH-EFV–gln were present in CSF at 2- to 9-fold higher concentrations than 8OH-EFV. The potential contribution of known and previously unreported EFV metabolites in CSF to the neuropsychological effects of efavirenz need to be further examined in larger cohort studies

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INTRODUCTION

Efavirenz (EFV) (**A**, in Figure 1) presents a large interindividual pharmacokinetic variability that can be attributed to differences in its metabolism (Csajka et al., 2003). A significant percentage of individuals under the current standard EFV dosage (600mg once daily) experience neuropsychological toxicity while a reduced dosage of 400 mg QD has been recently shown to be equally efficacious with reportedly a more favorable tolerance profile (Group et al., 2014). EFV tolerability is a major concern and in fact represents the main reason for premature antiretroviral treatment discontinuation (Lubomirov et al., 2011). Several studies have investigated the relationships between elevated EFV concentrations, genetic background and neuropsychological effects which have yielded controversial results as some associations have been found in some but not all studies (Marzolini et al., 2001; Gutierrez et al., 2005; Read et al., 2009; van Luin et al., 2009). In view of the conflicting results regarding the mechanism of EFV toxicity, the group of Tovar-y-Romo et al. have investigated *in vitro* the impact of EFV and two primary oxidized EFV metabolites on the function of primary neurones cultures and found that the metabolite 8-hydroxy-efavirenz (8OH-EFV) (**B**, in Figure 1) is a potent neurotoxin *in vitro* at concentrations one order of magnitude lower than those for EFV and 7-OH-EFV (Tovar-y-Romo et al., 2012). However, since this finding has as yet not been followed by a formal pharmacokinetic/dynamic analysis in patients, it remains unclear whether 8-hydroxy-efavirenz only can explain *in vivo* the neuropsychological disorders experienced by some patients on EFV.

In fact, EFV metabolic pathway is complex with different enzymes involved. EFV has three primary metabolites. It is mainly metabolized by CYP2B6 to 8OH-EFV, to a lesser extent by CYP2A6 to 7-hydroxy-efavirenz (7OH-EFV) (**C**, in Figure 1) and directly conjugated by UGT2B7 (Belanger et al., 2009; Kwara et al., 2009; Cho et al., 2011) to efavirenz N-glucuronide (EFV-N-gln) (**D**, in Figure 1). All primary metabolites are detected in humans although the abundance of EFV-N-gln in plasma is negligible and the contribution of the N-

glucuronidation pathway to the overall clearance of EFV appears to be minimal (Mutlib et al., 1999b; Kwara et al., 2009).

EFV has also secondary metabolites. The 8OH-EFV and 7OH-EFV are subjected to phase II metabolism *via* glucuronidation and/or sulfatation. The secondary metabolites 8OH-EFV–glucuronide (8OH-EFV-gln) (**E**, in Figure 1), 7OH-EFV–glucuronide (7OH-EFV-gln) (**F**, in Figure 1) and 7OH-EFV-sulfate (**I**, in Figure 1) metabolites have been identified in humans. However, the 8OH-EFV-sulfate (**H** in Figure 1) has been only detected in urine samples from rats and cynomologus monkeys (Mutlib et al., 1999b). The 8OH-EFV can also undergo subsequent metabolism to 8,14-di-hydroxy-efavirenz (8,14 di-OH-EFV) (**G**, Figure 1). The 8,14-di-OH-EFV has been barely detected in plasma (Avery et al., 2013). Mixed sulfate-glutathione di-conjugates of EFV have also been detected in humans (Mutlib et al., 1999a).

Genetic variability in the main (*CYP2B6*) and accessory EFV metabolic pathways (*CYP2A6*, *CYP3A4* and *UGT2B7*) can explain the differences in the metabolites profiles. (Rotger et al., 2007; di Iulio et al., 2009). Overall, the majority of the studies so far have been restricted to the phase I metabolites (Cho et al., 2011; Avery et al., 2013) even though, as we will show, these metabolites are present *in vivo* at much lower levels than phase II metabolites.

Our hypothesis is that some metabolites, instead of EFV itself, may be responsible for the neuropsychological toxicity associated with EFV treatment. In our studies, we have therefore investigated not only the phase I but also the major phase II metabolites that may also represent relevant phenotypic markers of the possible mechanism of this toxicity. Herein, we have performed a quantitative phase I and II metabolites profile analyses in plasma, cerebrospinal fluid (CSF) and urine samples from individuals under EFV therapy. We correlated the differences in the metabolites profiles with genetic variants in *CYP2B6* and *CYP2A6,* and examined the association between the concentrations of phase I and phase II EFV metabolites with both EFV treatment discontinuation and neuropsychological toxicity.

MATERIALS AND METHODS

Study population

A total of 71 plasma samples, 10 CSF samples and 8 urine samples from 71 participants were obtained from four study population subsets:

- Swiss HIV Cohort Study (SHCS) (Swiss et al., 2010) participants under EFV included in our previous study to evaluate the association of pharmacogenetic markers with time to treatment discontinuation during the first year of antiretroviral therapy (ART). A total of 39 plasma samples were selected according to their genotype (Lubomirov et al., 2011).

- SHCS participants that presented extremely high EFV plasma levels during routine Therapeutic Drug Monitoring (TDM) (n= 13 plasma samples).

- SHCS participants included in a previous study to evaluate the activity of monotherapy in the central nervous system (MOST study) (Gutmann et al., 2010) who were receiving EFV during the control period of the trial. (n=9 plasma and n=9 CSF samples). Among the CSF and plasma samples collected, six paired CSF-plasma samples were analyzed.

- During the routine medical follow-up, 8 SHCS patients , under stable EFV regimen (600mg once a day) agreed to donate a urine spot at the same time a TDM blood sample was collected during their usual medical follow-up visit (n=8 urine and n=8 plasma samples). These patients had normal hepatic and renal functions. They had undectable viremia at the moment of the visit and no toxicity was reported in these patients except for one individual who stopped the treatment because of CNS toxicity.

- One special case of a patient with extreme EFV plasma levels for whom a paired CSF-plasma sample was also collected during the investigation of very severe neuropsychological toxicity (Anagnostopoulos et al., 2013).

Samples were retrieved from the SHCS repository and/or from individuals treated with EFV followed in the routine TDM program for antiretrovirals at Lausanne University Hospital (CHUV), Lausanne.

Genotype data

Genotype data was obtained from 67 participants who were genotyped in the context of previous studies (Hasse et al., 2005; Rotger et al., 2005; di Iulio et al., 2009; Lubomirov et al., 2011; Anagnostopoulos et al., 2013) or for the present study for 6 decrease or loss of function variants in *CYP2B6* (n= 4), *2A6* (n= 1) and *3A4* (n= 1) All participants gave written informed consent for genetic testing.

In the present study, genotyping was performed by TaqMan allelic discrimination (Applied Biosystems, Foster City, California, USA): C_7817765_60 for rs3745274 (*CYP2B6*6*), C_33845811_20 for rs35303484 (*CYP2B6*11*), C_33845840_20 for rs35979566 (*CYP2B6*15*), C_30634332_10 for rs28399433 (*CYP2A6*9*) and C_32306227_10 for rs4646437 (*CYP3A4).* The rs28399499 (*CYP2B6*18*) was analyzed by PCR and direct sequencing. For the 13 participants with extremely high EFV plasma levels, *CYP2B6* and *CYP2A6* were fully re-sequenced (Rotger et al., 2007; di Iulio et al., 2009).

Participants were classified into 6 genetic scores according to their *CYP2B6, 2A6* and *3A4* genotype status as previously described (Lubomirov et al., 2011) and categorized in 3 groups, namely group 1: score 1, individuals homozygous for the reference alleles of *CYP2B6* and all accessory pathways; group 2: scores 3 and 4, individuals heterozygous for a loss-of-function (LOF) *CYP2B6* and/or at least one LOF allele in the accessory pathways; and group 3: score 5 individuals homozygous for a LOF in *CYP2B6* and score 6, individuals homozygous for a LOF in *CYP2B6* and at least one LOF allele in the accessory pathways (Rotger et al., 2007; di Iulio et al., 2009).

Chemicals and reagents

Efavirenz, 7OH-EFV, 8OH-EFV and 8,14-di-OH-EFV, (Figure 1 **A**, **B**, **C** and **G**) as well as their stable isotopically-labelled internal standards were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Sulfatase, β-glucuronidase, D-saccaric acid 1,4-lactone (D-SL; a specific glucuronidase inhibitor) was obtained from Sigma-Aldrich (St Louis, MO).

Enzymatic hydrolysis of plasma, urine and CSF

A 100 µL-aliquot of patient's sample (plasma, urine and CSF) was mixed with 300 µL of acetonitrile and centrifugated at +4ºC for 10 min at 20'000 g (14'000 rpm) on a Mikro 200R Hettich Benchtop centrifuge, Bäch, Switzerland). The supernatants (150 µL) were carefully transferred to a propylene tube and evaporated to dryness under a stream of nitrogen at room temperature (RT) to yield a solid extract residue that was reconstituted in a suitable buffer (see below) prior to the enzymatic treatment. For each enzymatic reaction, a control was prepared in parallel in the same conditions (without enzyme).

- *Sulfatase treatment:* Prior to incubation, solid sample extract residues were constituted with 70 µL of buffer (acetate ammonium 20 mM) and 30 µL of sulfatase from *Aerobacter aergenus* (3.1 mg protein/mL). 50 µL of a solution of D-saccarid acid 1,4 lactone (D-SL, a specific glucuronidase inhibitor) at 4 mg/mL in H_2O was added to inhibit the small quantity of glucuronidase (<10 UI/mL) present in the sulfatase enzyme. After 18 hours incubation in a water bath at 37ºC, the enzymatic reaction was terminated by adding 80 µL of internal standards solution containing EFV-d5, 7OH-EFV-d4 and 8OH-EFV-d4 at 300ng/mL in MeOH. After centrifugation of the reaction mixture at 14000 rpm, 150 µL of supernatant was transferred into HPLC vial and 20 µL was injected for the analysis by LC-MS/MS.

- *Glucuronidase treatment:* 20 µL of β-Glucuronidase from *Helix promatia* type HP-2 and 130 µL de 20 mM acetate (pH adjusted to 4.7) were added to sample extract residue. The mixture is vortexed-mixed for 5 sec and incubated at 37ºC in a water bath for 18h. A volume of 100 µL of internal standards solution in MeOH was used to

stop the glucuronidase reaction. After centrifugation (10 minutes, at 14000 rpm) the supernatant was transferred into HPLC vial and 20 µL was subjected to LC-MS/MS analysis.

Mass spectrometry analysis

The liquid chromatography system consisted of Rheos 2200 quaternary pumps, equipped with an online degasser and a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) controlled by Janeiro-CNS software (Flux Instruments, AG, Thermo Fischer Scientific Inc., Waltham, MA). Separations were done on a 2.1mm×50mm Atlantis® dC18 3mm analytical column (Waters, Milford, MA, USA). The chromatographic system was coupled to a triple stage quadrupole (TSQ) Quantum Ion Max mass spectrometer (MS) from Thermo Fischer Scientific, Inc., equipped with an electro-spray ionization (ESI) interface and operated with Xcalibur 2.0 software (Thermo Fischer Scientific Inc., Waltham, MA).

The phase mobile used for chromatography was 2 mM ammonium acetate in ultrapure water containing 0.1% formic acid (FA) (pH 3.5). Solution B consisted of 0.1% formic acid in ACN. The mobile phase was delivered using the following stepwise gradient elution program: 25% of B at 0 min to 40% at 6 min following by an isocratic mode at 40% of B until 10.5 min, then the percentage were gradually increased from to 60% of B over 14 min with a flow rate of 0.3 mL/min. The second part of the run includes 2 min of intensive rinsing (100% B with 0.5 mL/min) and re-equilibration step to the initial solvent up to 20 min. The autosampler was maintained at 10 °C. The volume injection was 20 µL. ESI in negative mode was carried out at a capillary temperature at 350 °C, the ESI spray voltage was set at 4 kV, and the source-induced dissociation was set at 10 V. The sheath and auxiliary gas (nitrogen) flow-rate was set at 35 and 10 (arbitrary units), respectively.

The phase II metabolites were identified by detailed mass fragmentation analysis: full scan, neutral loss, precursor ion scan and product ion scan. Precursor ion (Q1) scan data obtained

under negative ESI conditions *m/z* 330 for 7OH-EFV and 8OH-EFV, *m/z* 314 for EFV, and *m/z* 346 8,14-di-OH-EFV. Product ion scan (Q3) spectral data of the known synthetic metabolite standards were compared with those metabolite peaks obtained in plasma and urine samples. In neutral loss analysis, Q1 was fixed at m/z 410 and the loss was fixed at *m/z* 80. The hydroxyled efavirenz metabolites were identified by mass spectra comparison with previously published data (Mutlib et al., 1999a; Mutlib et al., 1999b) as well as by enzymatic conversion to the corresponding hydroxylated efavirenz metabolites.

The identity of the newly identifed metabolite 8OH-EFV sulfate in human was further confirmed by High Resolution (HR) mass spectrometry analysis using a Thermo Q-Exactive instrument equipped with the Orbitrap® HR technology, in the positive and negative mode. MS data processing was carried out using the Xcalibur software

Phase II metabolites quantification

Metabolites profiles analysis were performed by LC tandem MS in paired samples subjected, or not, to enzymatic hydrolysis. The concentrations of phase II metabolites have been calculated as the difference between total (i.e. after enzymatic hydrolysis) and direct determination of corresponding phase I metabolites (i.e. 7OH-EFV and 8OH-EFV, for which pure standards and d-4 labelled I.S. are available for quantification). The concentrations of the glucuronides and sultates metabolites in ng/mL has been obtained by multiplying with the coefficient 1.48 and 1.24 respectively, which corresponds to the ratio of the molecular weights (MW) of the respective phase II metabolites, divided by the MW of phase I hydroxy-metabolites (330 g/mole). EFV and metabolites concentration in urine have been normalized with creatinine urinary levels in mMole.

Statistical analysis:

Statistical analyses were performed using Prism 6 (version 6.04). Association between EFV and its metabolites concentration and genetic score or treatment continuation groups was

evaluated on $\log_{10}$-transformed data using t-test and *P* values of < 0.05 were considered statically significant.

RESULTS

Efavirenz metabolites profile analysis.

Typical chromatographic analysis by LC-MS/MS using the Selected Reaction Monitoring (SRM) of EFV and its hydroxy-metabolites in a plasma extract sample is shown in Figure 2. The third chromatographic profile in Figure 2 shows the presence of signals at m/z 314+16 (330) indicative of at least three major mono hydroxy-metabolites, either directly present in plasma or possibly produced during the spray/ionisation step of the MS analysis (i.e. in-source metabolite dissociation). The metabolite 8OH-EFV was unambiguously identified based on comparison of its retention time (RT=11.8 min), precursor ion scan data, and MS/MS fragmentation pattern with synthetic metabolite standard. However, 7OH-EFV (RT=10.5 min) was not (or barely) detected in participants' plasma samples. Two additional as yet unidentified peaks at RT= 6.1 (= Y) and 9.3 min (=X) were also detected on the *m/z* transition signal selected for mono-hydroxylated EFV metabolites. We hypothesized these signals were the result of a possible *in-source* metabolite dissociation, and the molecular weight of some putative phase II metabolites of hydroxy-efavirenz (mw=330) were screened, namely, the glucuronide (mw=490) and sulfate (mw=410).

Identification of the metabolite 8OH-EFV sulfate

The mass spectrum of the hydroxy metabolite Y eluted at 6.1 min was identified as the EFV-O-glucuronide because of the in-source dissociation of the glucuronide. This peak identification was confirmed in a separate sample analysis by single ion monitoring at [M-H]$^-$= 506 corresponding to the molecular weight of EFV–O-gln (MW 507).

The mass spectrum of the hydroxy metabolite X eluted at 9.3 min obtained by full scan analysis between m/z 100-600 is shown in Figure 3A. It had a molecular weight of 410, compatible with a sulfate of either 7OH-EFV or 8OH-EFV. Full scan mass analysis of

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compound X and its comparison with published mass spectra data (Mutlib et al., 1999a; Mutlib et al., 1999b) allows the unambiguous assignment of the metabolite as being the metabolite 8OH-EFV-sulfate, identified for the first time in humans. This metabolite was differentiated from the known 7OH-EFV-sulfate by comparison of their mass spectral fragmentation pattern. While the ESI-MS/MS of X showed a signal [M+H]- at m/z 410 for the molecular weight of sulfate conjugates of both 8OH-EFV and 7OH-EFV, we observed an important fragment ion for the aglycone at *m/z* 330 which is the characteristic signature for 8-hydroxylated analogues of E (Mutlib et al., 1999b). For comparison in Figure 3B, this signal at *m/z* 330 is actually missing in the mass spectrum of the sulfate conjugate of 7OH-EFV that could be also detected at 7.5 min in the metabolites profile analysis monitored at *m/z* 410 (Figure 4A). The enzymatic hydrolysis of plasma by sulfatase results in a corresponding increase in the respective minor 7OH-EFV and major 8OH-EFV metabolites and confirms the major 8-substituted hydroxy-sulfate conjugate metabolite identification.

Finally, the HR mass spectrum of 8OH-EFV-sulfate shows a signal at m/z [M-H]$^-$ = 409.97216 in excellent correspondance to the calculated molecular mass of (m/z [M-H]$^-$ = 409.97075 . The difference between observed and calculated masses were < 5ppm providing high confidence in the providing a definitive identity assessment

Quantification of Phase I and II metabolites of efavirenz in plasma, CSF and urine.

Total plasma concentrations of EFV and the six metabolites determined in the various body fluids from 71 participants prior to, and after enzymatic treatment with glucuronidase and sulfatase (in the presence of the glucuronidase inhibitor D-SL) are shown in Figure 5 A and reported in Table 2. Considering the entire population, the 8OH-EFV–glucuronide and the 7OH-EFV–glucuronide were the major metabolites circulating in plasma (Table 2) with median (range) concentrations of 5827 ng/mL (414-17107) and 949 ng/mL (0-11797). 8OH-EFV-Gln is 64 fold higher than the parent 8OH-EFV (figure 5A). The median plasma levels of the newly identified metabolite 8OH-EFV-sulfate was 771 ng/mL (0-12611), 7-fold higher than the parent 8OH-EFV metabolite, while the plasma concentration of 7OH-EFV sulfate

was 269 ng/mL (0-3019) (figure 5A). The metabolite-to-EFV ratios in plasma, expressed in %, are shown in Figure 5B and reported in Table 2. Interestingly, the median ratio % of 8OH-EFV-Gln/EFV is greater than 200 with plasma concentrations of 8OH-EFV-gln exceeding the level of EFV in 70 % of cases. Finally, the 8,14-di-OH-metabolite of EFV was essentially not detectable in plasma.

EFV concentrations obtained after the reanalysis of CSF samples were greater than 0.5 ng/mL (the suggested CSF 50% maximal inhibitory concentration (IC_{50}) for wild-type HIV virus (Best et al., 2011) in all samples. The median concentration of EFV in CSF was 16 ng/mL (range 9-31), besides the exceptional value at 143 ng/mL measured in the individual with severe neuropsychological toxicity (Anagnostopoulos et al., 2013) (Figure 5C, closed square). The median concentration of the phase I metabolite 8OH-EFV in CSF was 4 ng/mL (range 0-11). In 6 out of 10 samples, the 8OH-EFV concentrations in CSF were higher than the reported toxicity threshold of 3.3 ng/mL associated with dendritic damage as suggested by Tovar-y-Roma et al. in their *in vitro* study (Tovar-y-Romo et al., 2012). Both 7OH-EFV and 7OH-EFV-sulfate were below the Limit of Quantification (LOQ) of 2 ng/mL of our assay (Best et al., 2011). By contrast, 8OH-EFV–gln, 8OH-EFV–sulfate and 7OH-EFV–gln were present in CSF at concentration comprised within 15-56, 0-29 and 0-13 ng/mL, respectively (Figure 5C). Overall, these phase II metabolites were present in CSF at about 2 to 9 fold higher concentrations than 8OH-EFV. The median CSF/plasma ratios (%) (n=7) were 1.5, 2.5, 0.45, 1.25 and 0.44% for EFV, 8OH-EFV, 8OH-EFV–gln, 8OH-EFV–sulfate and 7OH-EFV–gln, respectively (Figure 5D).

The results of analysis of urine samples are shown in Figure 5E with EFV and metabolites levels normalized with creatinine urinary levels. The 7OH-EFV-sulfate was consistently the principal urinary sulfate conjugate (Figure 5E). Finally, 8,14-di-OH-sulfate was found in significant amount in urine (data not shown).

Phase I and II metabolites profiles in plasma and CSF from genotyped participants

Among the 67 participants with genotype data, 33% were classified in the group 1, 18% in the group 2 and 49% in the group 3.

The metabolites-to-EFV concentration ratios in plasma in these genotyped participants are shown in Figure 6. The median log (8OH-EFV/EFV) ratio was 0.8, 1.1 and 0 % in group 1, 2 and 3, respectively. As expected, the 8OH-EFV/EFV ratio was significantly lower ($p < 0.0001$) in group 3 as compared to the other groups.

The log-transformed 8OH-EFV–gln/EFV and 8OH-EFV-sulfate/EFV ratios in the three groups parallel those determined for 8OH-EFV/EFV. In comparison to group 1 and 2, group 3 individuals had significantly lower median log-transformed 8OH-EFV–gln /EFV ratios (2.7, 2.7 and 2 %, respectively, $p < 0.0001$) and log-transformed 8OH-EFV-sulfate/EFV (1.8, 1.8 and 1%, respectively ($p < 0.0001$).

To evaluate the contribution of the accessory pathways (CYP2A6 and CYP3A4) in overall 7OH-EFV metabolite abundance in plasma samples, log-transformed ratios from individuals with score 5 (Ref for both *CYP2A6* and *CYP3A4*) *versus* score 6 (Decrease of Function (DOF) for *CYP2A6* and/or *CYP3A4*) were compared. The median log transformed 7OH-EFV-gln/EFV and 7OH-EFV-sulfate/EFV ratios (%) were 1.6 *vs* 1.5, and 0.9 *vs* 0.9, respectively.

Exploratory analyses were carried out to examine whether hydroxy metabolites levels in CSF are influenced by genetic status. Among 10 CSF samples, 6 were obtained from genotyped participants, who were classified into (score 1, n=2), (score 4, n=3) and (score 6, n=1). Median CSF levels of 8OH-EFV, 8OH-EFV–gln and 8OH-sulfate in score 1 and score 3, 4 and 6 groups were similar: 7 vs 8, 30 vs 52, and 19 vs 16 ng/mL, respectively.

Of note, the paired sample from one individual with severe neuropsychological toxicity because of LOF/DOF alleles of *CYP2B6* (Anagnostopoulos et al., 2013) had extreme EFV concentrations of 46232 ng/mL and 143 ng/mL, in plasma and CSF, respectively (shown in

figure 5A and 5B, closed squares). In this individual, plasma levels of 8OH-EFV, 8OH-EFV–gln, and 8OH-EFV–sulfate concentrations were 37, 6895 and 1399 ng/mL, respectively, while the concentrations of 7OH-EFV, 7OH-EFV–gln, and 7OH-EFV–sulfate were 11, 6932, 1750 ng/mL, respectively (figure 5A, closed squares). In CSF, despite extremely high EFV levels, neither 8OH-EFV, nor 7OH-EFV and 7OH-EFV-sulfate were detected while the concentration of 8OH-EFV–gln, 8OH-EFV sulfate or 7OH-EFV-gln were 15, 11 and 12 ng/mL, respectively.

Phase I and II metabolites profiles, early treatment discontinuation and CNS effects

Data on EFV treatment discontinuation or dose reduction was available from our previous study (n= 39) (Lubomirov et al., 2011) or was retrieved from the SHCS (n= 29). A total of 51 of 68 participants discontinuated the EFV based therapy. In the present analysis, neither EFV nor any of its primary and secondary metabolites were found to be associated with increased risks of EFV treatment discontinuation (Supplemental Figure 1).

Among the 51 patients who discontinued efavirenz-based treatment, the reason for discontinuation was reported in 42 patients: 15 patients stopped the treatment because of neurological (CNS) toxicity and 27 patients discontinued the therapy for other reasons (non-neurological reasons, i.e. gastrointestinal toxicity, treatment failure, patient own wish, etc…). As shown in figure 7, no difference was observed in the plasma levels of EFV nor 8OH-EFV or any secondary metabolites in individuals who developed CNS toxic effects or who stopped therapy for other reasons.

Among the 15 patients who stopped EFV treatment because of CNS toxicity, the majority (11/15, 73%) had a decreased (score 4, n=1) or loss of function (score 5 and 6, n=10) genotype for the major metabolic pathway CYP2B6, in line with previously reported results by Rotger et al.(Rotger et al., 2005). Finally, we examined data from patients with CSF-plasma paired samples: among the 3 out of 7 patients with available CSF who have developed CNS disorders and stopped EFV therapy, no difference was observed in the CSF concentrations of EFV, 8OH-EFV nor any of its metabolites.

DISCUSSION

We have performed a comprehensive quantitative metabolite profile analysis of EFV comprising both phase I and phase II metabolites in plasma, CSF and urine.

In plasma, phase I metabolites were present at rather low levels (up to 200 ng/mL) in accordance with previously published data (Cho et al., 2011; Avery et al., 2013) whereas the phase II metabolites (8OH-EFV–gln, 7OH-EFV–gln and 7OH-EFV-sulfate) were found at comparable or even higher levels (up to 17107 ng/mL) than EFV itself. We also identified for the first time in humans the 8OH-EFV-sulfate that was found at higher levels than the known 7OH-EFV-sulfate. Previous reports had reported of 8OH-EFV-sulfate in urine samples from rats and cynomologus monkeys (Mutlib et al., 1999b). The identification of the new metabolite in humans may have consequences if EFV is used as a probe to phenotype the CYP2B6 activity (Rakhmanina et al., 2012). In previous studies only the glucuronide of 8OH-EFV was considered as the single mono hydroxy-EFV conjugate metabolite (Mutlib et al., 1999b). In the future, not only 8OH-EFV-gln, but also 8OH-EFV-sulfate will have to be considered and hydrolysed accordingly to yield 8OH-EFV. The 8,14-di-OH-metabolite was almost undetectable in plasma. Recent *in vitro* investigations have shown that the 8,14-di-OH-EFV metabolite was not produced when the 8OH-EFV was directly incubated in human liver microsomes (Ogburn et al., 2010). This finding is also in agreement with the absence of the 8,14-di-OH-EFV in patients' plasma. However, the sulfate of the 8,14-di-OH-EFV was found in significant amount in urine, suggesting that 8OH-EFV may first undergo a phase II conjugation step (i.e. sulfation, or glucuronidation) prior to the second hydroxylation at position 14 (Ogburn et al., 2010).

In CSF, 8OH-EFV levels found in our study were in close correspondence to those reported previously (Avery et al., 2013; Winston et al., 2014). 8OH-EFV, 8OH-EFV–gln, 7OH-EFV–gln and 8OH-EFV-sulfate were detected in CSF with 8OH-EFV–glucuronide and 8OH-EFV-sulfate being the major metabolites, the latter with a median CSF/plasma ratio of 1.25%, similar to that of EFV (1.5%). These observations were unexpected with regards to

the enhanced polarity of the metabolites that *a priori* should reduce their passage across the lipophilic blood brain barrier. These findings are reminiscent of what has been observed for the archetypal example of morphine and its metabolite morphine-6-glucuronide (Mo-6-gln) (De Gregori et al., 2012). This latter metabolite, besides its "high polarity" has been also reported in some (Huwyler et al., 1996; Sattari et al., 2011), but not all studies (Bourasset et al., 2003), to be a substrate of P-glycoprotein that should decrease further its blood brain barrier permeability. Additional carrier-mediated processes have been found to determine Mo-6-gln (and morphine) neuropharmacokinetics and brain distribution (Bourasset and Scherrmann, 2006). The transporters implicated for the active transport of Mo-6-gln accross blood brain barrier include GLU-1 and a digoxin-sensitive transporter (probably oatp2) (Bourasset et al., 2003). Interestingly, despite a lower uptake and reduced blood brain barrier permeability as compared to morphine (Wu et al., 1997), Mo-6-gln has now been recognized to play a large, if not the major, role for the analgesic effect after morphine administration to patients (Klimas and Mikus, 2014; Mikus and Klimas, 2015). By analogy with Mo-6-gln, further *in vitro* studies in cell systems are warranted to clarify the potential role of transporters for both the unexpected blood brain barrier passage observed for 8OH-EFV–gln and 7OH-EFV–gln, their overall brain distribution, and possibly neurotoxicity.

In urine, 7OH-EFV-sulfate was the most abundant metabolite showing the opposite relative proportion with the plasma samples wherein 8OH-EFV-sulfate was the principal sulfate conjugate. It is unclear whether this is due to a possible difference in renal clearance of sulfate conjugates although results should be interpreted with caution with the limited number of urine samples available.

We have examined the relationships with genetic variants in the main (*CYP2B6*) and accessory (*CYP2A6* and *CYP3A4*) EFV metabolic pathways and the different metabolites. Individuals homozygous for LOF alleles in *CYP2B6* presented the lowest 8OH-EFV metabolites-to-EFV concentration ratio that can be considered as a phenotypic index of CYP2B6 functionality. The reduced capacity to hydroxylate EFV at position 8 was observed

not only at the primary phase I reaction, but was also reflected in the subsequent glucuronidation and sulfation steps. For instance, in the patient with LOF/DOF alleles of *CYP2B6* (Anagnostopoulos et al., 2013), the plasma concentration of 8OH-EFV was amongst the lowest encountered in our study. Consistent with this, the corresponding 8OH-EFV sulfate plasma level was also very low while the 8OH-EFV-gln levels were in the lower range. The phenotypic activity of CYP2A6 that catalyses 7-hydroxylation-EFV (Ogburn et al., 2010) could not be directly ascertained using 7OH-EFV since in our study this metabolite was found to be present in plasma at levels mostly under the LOQ. Indirect comparison of CYP2A6 activity may be made *via* the analysis of subsequent phase II metabolites of 7OH-EFV. The 7OH-EFV-gln/EFV and 7OH-EFV sulfate/EFV ratios were however similar in score 5 and score 6 groups, which challenges our current knowledge on the EFV 7-hydroxylation mediated by CYP2A6 (Ogburn et al., 2010). It may be argued that the variability of the secondary glucuronidation and sulfation steps would obscure the impact of the primary CYP2A6-mediated 7-hydroxylation of EFV *in vivo*. Nevertheless, the patient with LOF/DOF alleles of *CYP2B6* and extreme neurotoxicity requiring admission in a psychiatric hospital (Anagnostopoulos et al., 2013) had very low 7OH-EFV level in plasma (as most LOF study participants) but had the highest 7OH-EFV-gln levels, in plasma and CSF, and amongst the highest plasma concentration of 7OH-EFV-sulfate in our study. This is consistent with our proposed compensatory CYP2A6-mediated mechanism (di Iulio et al., 2009) occurring when CYP2B6 pathway is impaired, assuming that any formed 7OH-EFV is readily subjected to subsequent conjugation with glucuronide and sulfate.

We did not observe a correlation between metabolite levels and EFV treatment discontinuation because of CNS toxicity. In particular, the metabolite 8OH-EFV, which was reported to be a potent neurotoxic *in vitro,* with an effect distinct from EFV, is exactly the metabolite that is the minimally produced when CYP2B6 pathway is impaired, and remains overall at relatively constant, low level both in plasma and CSF, regardless of *CYP2B6* genotypes. This is an intringuing finding as patients' *CYP2B6* genotype *per se* was found to be a predictor of EFV neurotoxicity, in line with previous data (Rotger et al., 2005). Thus, one

must at least assert that 8OH-EFV is certainly not the single likely candidate for CNS toxicity and other metabolites have therefore to be considered.

In conclusion, we have performed a comprehensive quantitative metabolite profiles analysis of EFV in plasma, CSF and urine, and examined their relationships with the presence of certain genetic variants in *CYP2B6* and *CYP2A6 and CYP3A4* and with treatment discontinuation because of CNS toxicity. During these investigations, we have identified the new metabolite 8OH-EFV-sulfate present at high concentrations in all body compartments. More generally, we have demonstrated for the first time that the dominant circulating (as well as in CSF and urine) metabolites of EFV are not its primary oxidized products (e.g. 7-OH- and 8-OH-EFV) but rather the downstream phase II EFV metabolites (glucuronide and sulfate) of the product of EFV oxidation by *CYP2B6* and *CYP2A6*, some of them attaining concentrations even higher than those for EFV itself. We have observed that phase II EFV metabolites were the principal metabolites present in CSF, an unexpected finding given the higher polarity of glucuronide and sulfate as compared to the lipophilic parent compound EFV. The clinical importance of these previously unreported EFV metabolites in CSF and their potential contribution to the neuropsychological effects of efavirenz need to be examined in larger cohort studies.

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AUTHORSHIPS CONTRIBUTION

Contributed new reagents or analytic tools: H. Hugues, A. Anagnostopoulos, S. Yerly, P. Vernazza, H.F. Günthard, Swiss HIV Cohort Study

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Wrote or contributed to the writing of the manuscript: M. Aouri, C. Barcelo, M. Rotger, A. Telenti, L.A. Decosterd.

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Footnotes:

FIGURES LEGENDS

Figure 1: Principal phase I and II efavirenz metabolites A. Efavirenz (EFV); B. 8OH-EFV; C. 7OH-EFV; D. EFV–N-glucuronide (gln); E. 8OH-EFV-gln; F. 7HO-E-gln; G. 8,14 di-OH-EFV; H. 8OH-EFV-sulfate; I. 7OH-EFV-sulfate.

Figure 2: Typical profile SRM analysis chromatograms of EFV and its hydroxy-metabolites in plasma extract sample from one HIV-positive individual. Bottom: chromatographic profile of d4-labelled Internal standards of 7OH-EFV et 8OH-EFV.

Figure 3: Mass spectra of (A) 8OH-EFV-sulfate and (B) 7OH-EFV-sulfate, obtained using ESI in the negative mode, during the chromatographic separation as shown in Figure 4.

Figure 4: EFV metabolites profiling of plasma sample before (A) and after (B) sulfatase treatment. The first and second trace correspond *to m/z* 410 (hydroxy-EFV-sulfate) and *m/z* 330 →258 (mono-hydroxy-EFV), respectively. Bottom: chromatographic profile of pure standard of 7OH-EFV and 8OH-EFV.

Figure 5: (A) Plasma concentration of EFV and its metabolites (median + IQR 25-75), (B) Ratios of Metabolites/EFV in patients from all subset studies (median); (C) CSF from MOST study (n=9) and one CSF sample (closed squares) from neurological investigations (Anagnostopoulos et al., 2013) (median + IQR 25-75);(D) Ratio of paired samples CSF/Plasma from MOST study (n=6) and one CSF sample from neurological investigations (closed squares); (E) Metabolites profiles analysis of urine normalized with creatinine levels in urine (median + IQR 25-75) from a subset of consenting patients under stable efavirenz regimen, collected at the occasion of their regular medical follow-up visit, which includes TDM

Figure 6: Metabolites/EFV logarithmic ratios stratified according to functional status of CYP2B6 in 67 patients (Ref=reference; DOF=Decrease of Function; LOF=Loss of function).

Figure 7: EFV and 8OH-EFV-metabolites levels in plasma in individuals who had reduced dose or discontinued the EFV, classified according to central nervous system side effects (CNS) and other reasons of discontinuation (non neurological) (1).

TABLES

Table 1: Description of genetic risk scores for Efavirenz (Lubomirov et al., 2011) and distribution of genotyped patients (n=67) according to genetic scores.

Gene	Gene variant	Score 1	Score 2	Score 3	Score 4	Score 5	Score 6
CYP2B6	rs3745274 (*6)	0	0	1	1	2	2
	rs35303484 (*11)						
	rs35979566 (*15)						
	rs28399499 (*18)						
CYP2A6	rs28399433 (*9)						
CYP3A4	rs4646437	0	1 to 4	0	1 to 4	0	1 to 4
Number of Genotyped patients		22	0	5	7	15	18

Table 2: Concentrations (median and range) of EFV and its metabolites in plasma, CSF and urine in patient under EFV regimen

	Plasma				CSF				Urine	
	Concentrations (ng/mL) (n=71)		Metabolites /EFV ratios (%) (n=71)		Concentrations (ng/mL) (n=10)[a]		CSF/ Plasma ratios (%) (n=7)[b]		Urine/CRT ratios (n=8)	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
EFV	2100	326-46232	NA	NA	16	9-143	1.5	0.4-4.2	0.01	0.002-0.02
8OH-EFV	92	0-1086	3	0-66	4	0-11	2.5	0-4.6	0.15	0.073-1.13
8OH-EFV-gln	5827	414-17107	254	15-1607	35	15-56	0.45	0.28-1.5	19	3.610-29.80
8OH-EFV-Sulfate	771	0-12611	36	0-547	10	0-29	1.25	0-13.4	0.03	0.007-0.05
7OH-EFV	0	0-432	0	0-30	0	0	0	0-0	0.03	0.016-0.1
7OH-EFV-gln	949	0-11797	40	0-218	6.5	0-13	0.44	0-1.5	2.08	1.022-9.50
7OH-EFV-sulfate	269	0-3019	11.5	0-96	0	0	0	0-0	0.12	0.05-0.52

[a] EFV metabolite concentrations were determined in 10 samples

[b] Only 7 out of 10 CSF samples were available as paired CSF / plasma samples

CRT: creatinine urine levels [μmol/l]

Figure 1

Figure 2:
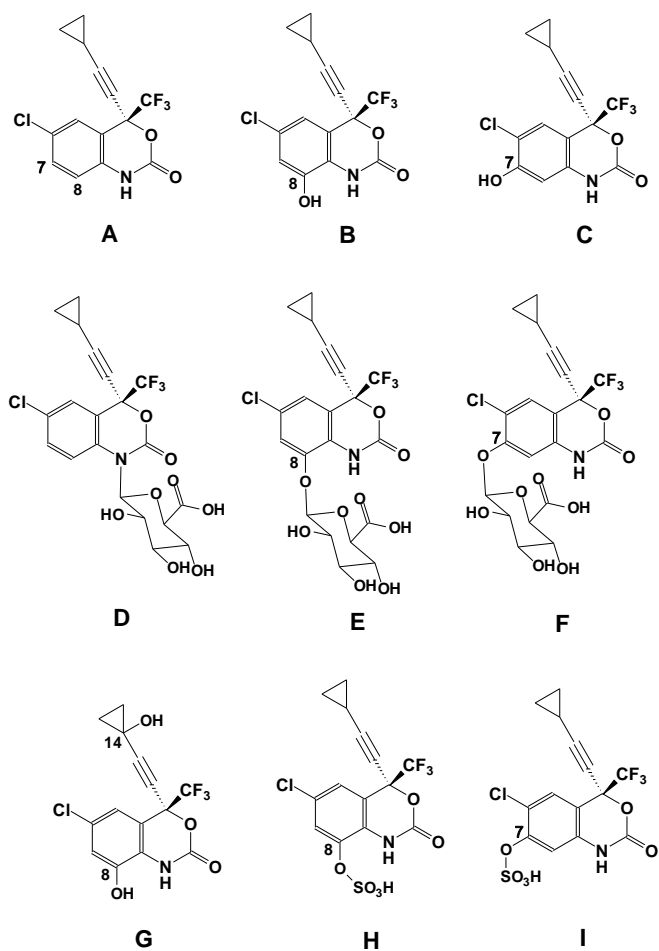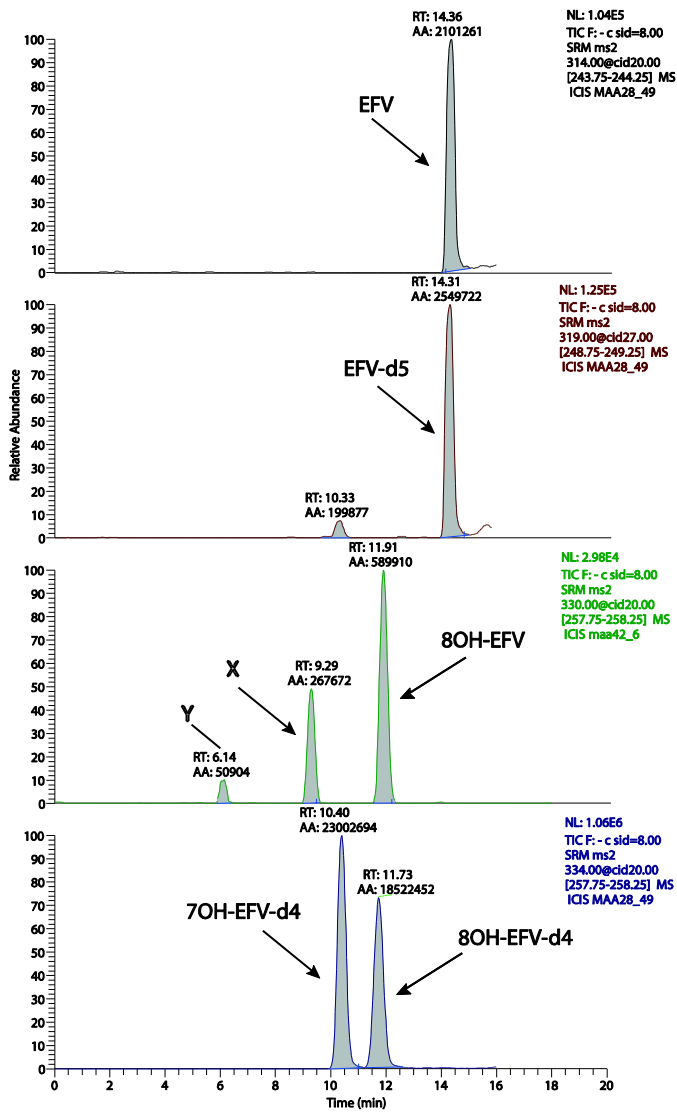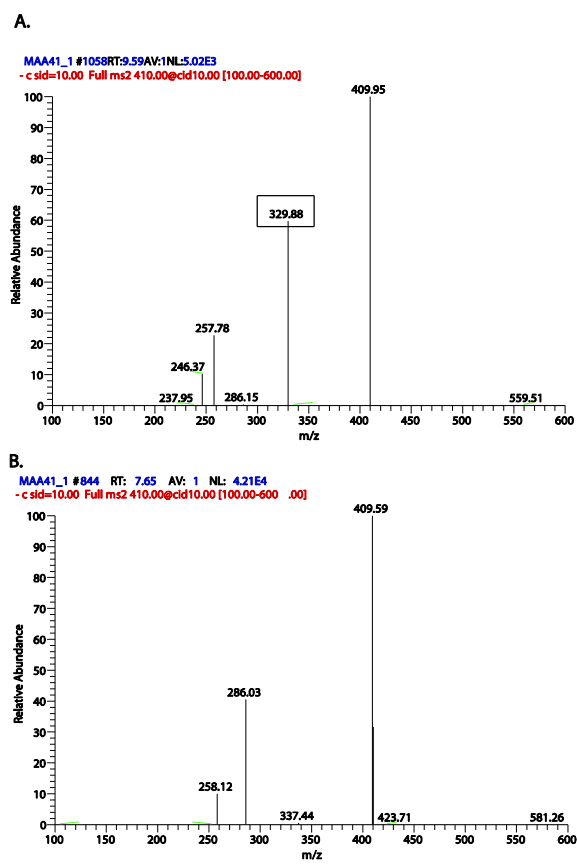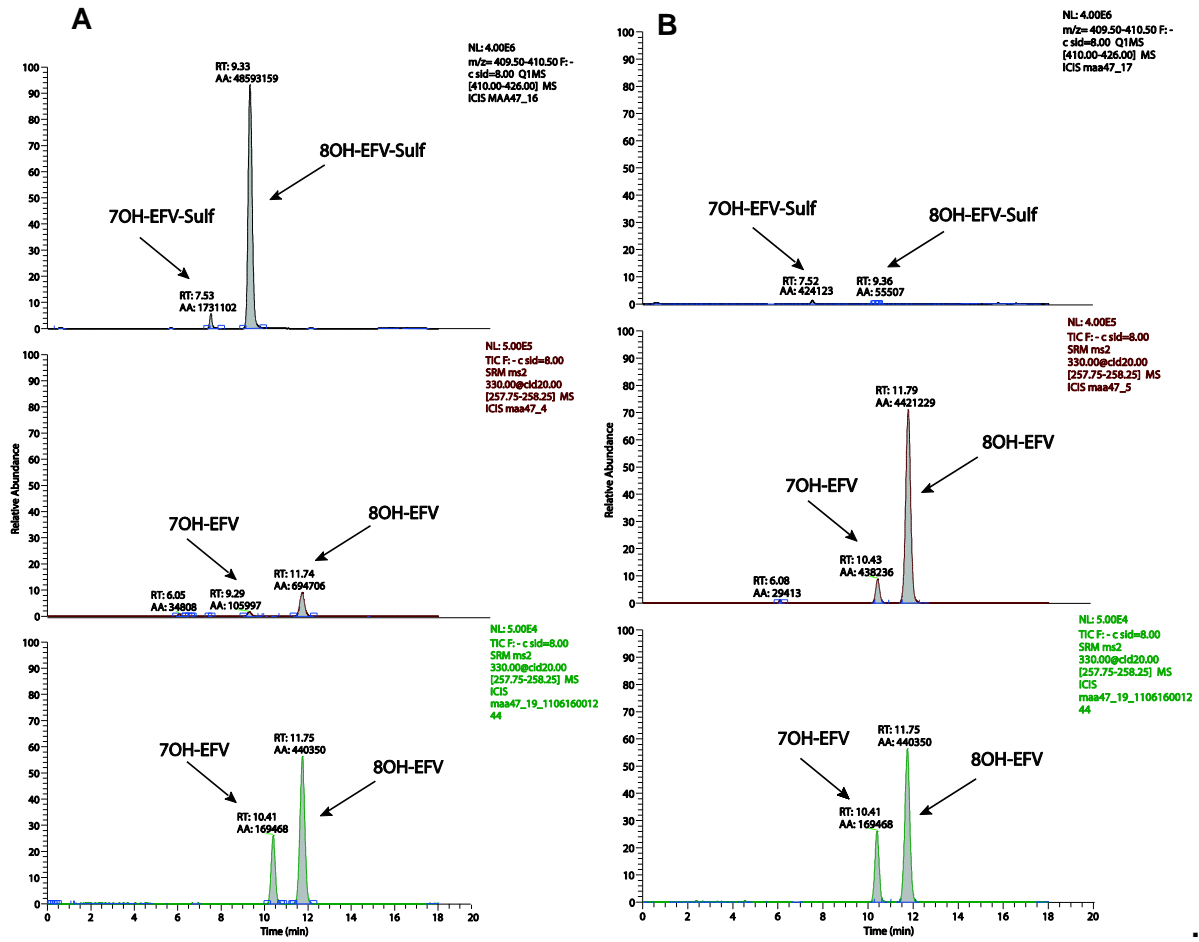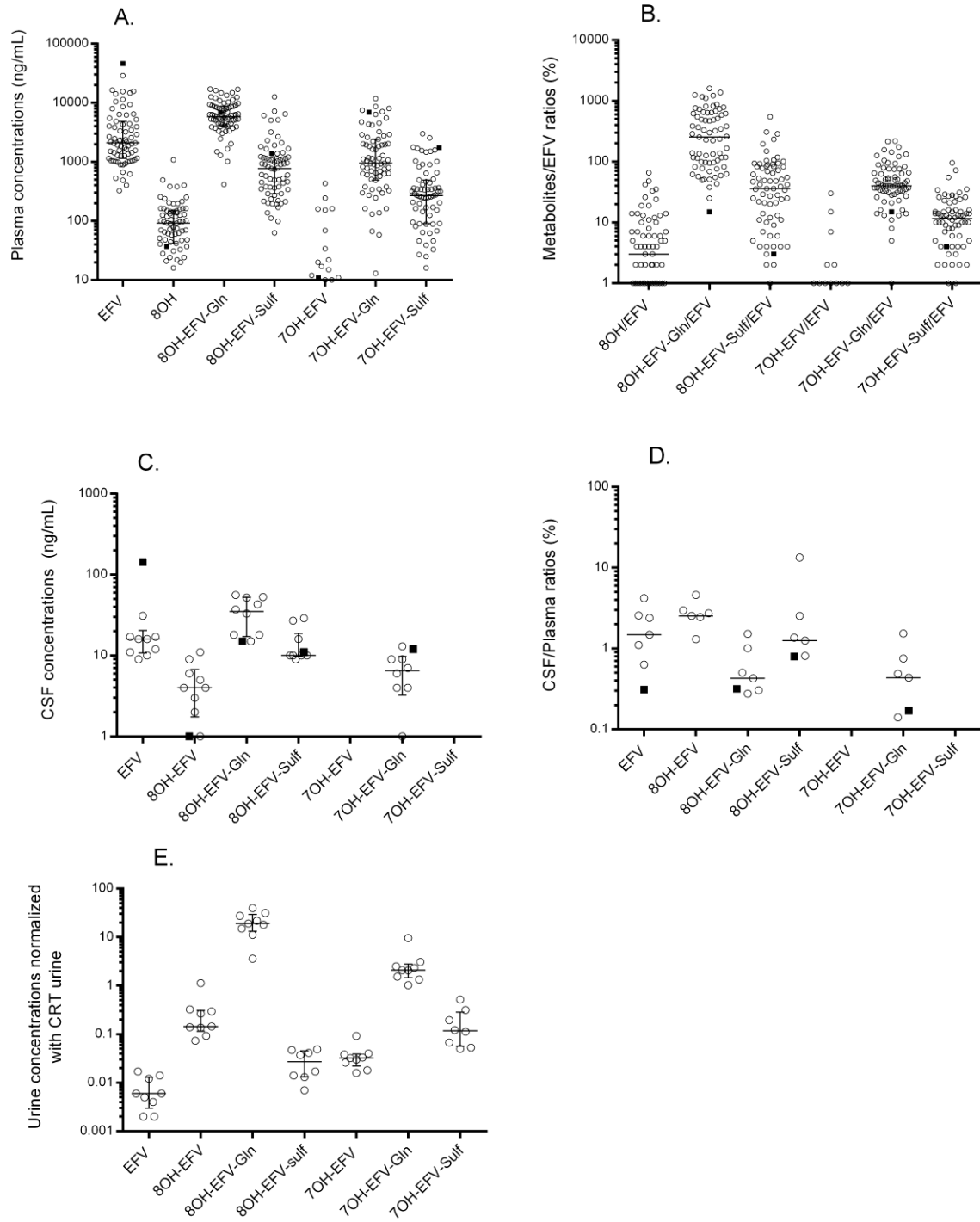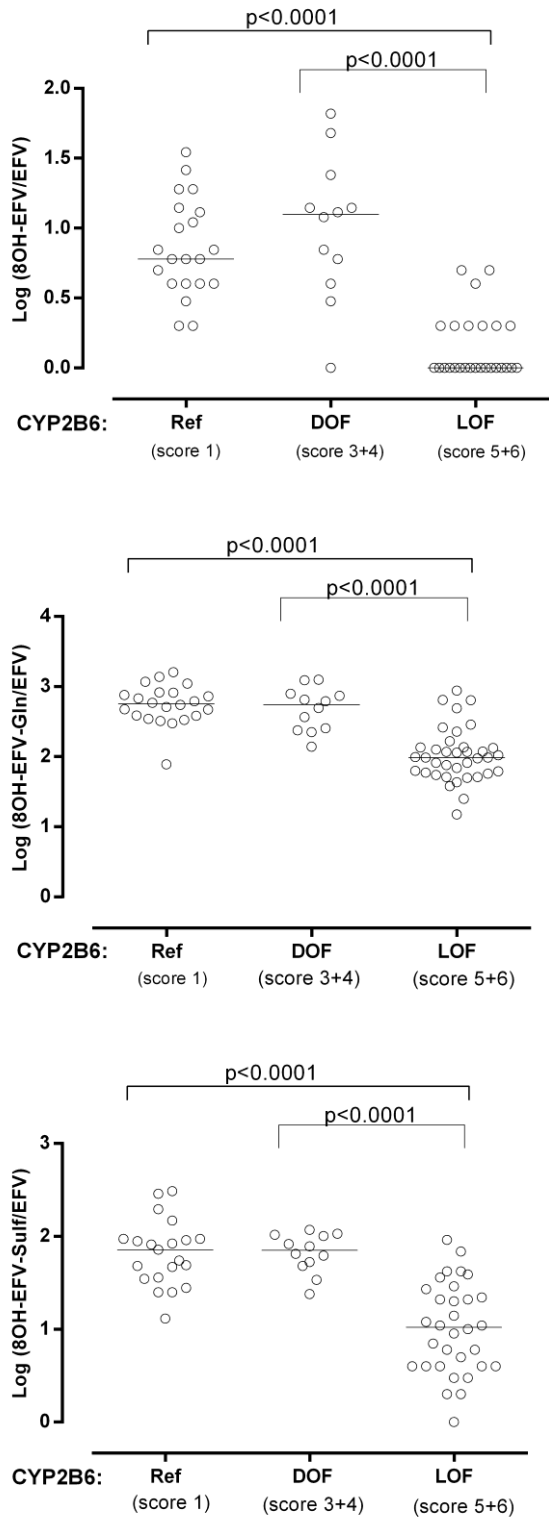
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Figure 4

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Figure 6

Figure 7