TITLE PAGE

Title: Effects of a novel *UGT2B* haplotype and *UGT1A4 *3* allele variants on glucuronidation of clozapine *in vivo*

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Running title: Effects of various *UGT* polymorphism on clozapine glucuronides

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ABSTRACT

Background: Glucuronidation is an important metabolic pathway of clozapine (CLZ), but the impact of various uridine 5'diphospho-glucuronosyltransferases (*UGT*) polymorphisms on the exposure and metabolism of CLZ *in vivo* is unclear.

Objective: The objective of this study was to investigate the impact of *UGT2B* haplotype and *UGT1A4*3* allele variants on the formation of CLZ glucuronide metabolites ($5N$- and N^+-glucuronide) and CLZ exposure in patients' serum after adjusting for sex, age and smoking habits.

Methods: The study was based on serum samples from CLZ-treated patients (n=79) subjected to routine therapeutic drug monitoring (TDM) at Diakonhjemmet Hospital, Oslo, Norway. From the same patients the following *UGT* variants were genotyped using Real-Time PCR: *UGT2B:GA* haplotype (defined as *UGT2B:GA*; *rs1513559A>G* and *rs416593T>A*) and *UGT1A4*3* (*rs2011425T>G*). Serum concentrations of CLZ $5N$- and N^+-glucuronide were measured by UPLC high-resolution mass spectrometry.

Results: None of the genotypes had significant impact on CLZ exposure (p>0.05). However, compared to *UGT2B:AT/AT* and *UGT1A4*1/*1*, the $5N$-glucuronide exposure was reduced in *UGT2B:GA/GA* carriers (-75%, p=0.03) while the exposure was non-significantly increased in *UGT1A4*3* carriers (+100%, p=0.14), respectively. The N^+-glucuronide exposure was unchanged in *UGT1A4*3 vs* noncarriers (p=0.28), but significantly reduced in heterozygous (-50%, p=0.016) and homozygous carriers (-70%, p=0.021) of *UGT2B:GA* compared to *UGT2B:AT/AT* carriers, respectively.

Conclusion: The *UGT2B:GA* and *UGT1A4*3* variants had no impact on CLZ exposure, but were associated with differences and preferences in CLZ glucuronidation. The latter might be of potential relevance for CLZ tolerability, since levels of the N^+-glucuronide metabolite may reflect the generation and trapping of reactive metabolites involved in CLZ-induced toxicity.

Keywords: Clozapine, Uridine 5'-diphospho-glucuronosyltransferase, Therapeutic drug monitoring, Glucuronidation, Metabolism, High-resolution mass spectrometry.

1. INTRODUCTION

Clozapine (CLZ) is the superior atypical antipsychotic drug with respect to symptom improvement in patients with schizophrenia [1]. The therapeutic use of CLZ is limited to patients with treatment resistant schizophrenia (TRS) due to the risk of serious CLZ-induced toxicity such as neutropenia, agranulocytosis and seizures [2, 3]. Despite this restriction in the use of CLZ, it is considered to be underutilized in the treatment of TRS patients [4]. A possible contribution to the observed underutilization may be the major interindividual variability in CLZ serum concentrations, which is challenging for obtaining an optimal dose within the narrow therapeutic range of 1071-1836 nM [5].

The metabolism of CLZ involves many enzymes, including several cytochrome P450 (CYP) enzymes and uridine 5'-diphospho-glucurosonyltransferases (UGTs) [6]. Of particular interest in CLZ metabolism are the CYP1A2, CYP3A4, CYP2D6, UGT1A and UGT2B enzymes [6, 7], in which the expressions of CYP1A2 and UGTs are induced by smoking [8, 9]. In two recent genome-wide association studies (GWASs), investigating associations between single nucleotide polymorphisms (SNPs) and *N*-desmethylclozapine (*N*-DMC)-to-CLZ ratios in patients' serum, significant loci were observed between the *UGT2B10/15* genes [10, 11]. The lead SNPs *rs1670747* and *rs2926038* were highly significant with p-values above 2.0 x 10^{-23} [10]. The minor allele frequencies (MAF) of *rs1670747* and *rs2926038* in the European population were 0.23 and 0.10 [12], respectively. The function of these SNPs beyond affecting CLZ metabolism is unknown. However, polymorphisms in the nearest genes, i.e. *UGT2B10* and *2B15* have been described to influence the glucuronidation rate of cotinine (nicotine metabolite) and postoperative anxiety after lorazepam premedication, respectively [13, 14]. Furthermore, much interests have been paid to the *UGT1A4*3* (142 T>G, L48V, MAF = 0.077) allele variant [15], which has been shown to alter the glucuronide formation of various compounds *in vitro* [15-18]. Particularly, the formation of CLZ 5*N*-glucuronide was increased in cell lines overexpressing *UGT1A4*3/*3*, suggesting an increased function phenotype of the *UGT1A4*3* allele variant in CLZ glucuronidation [19].

No studies have per date investigated the effect of the *UGT1A4*3* and *UGT2B* allele variants *(rs1670747* and *rs2926038)* on the glucuronidation of CLZ *in vivo*. The aim of the present study was therefore to investigate the impact of *UGT1A4*3* and *UGT2B* haplotype (based on *rs1670747G>A* and *rs2926038G>C*) variants on the formation of the two major glucuronides in human serum, i.e. CLZ 5*N*- and N^+-glucuronide, and potential effect on CLZ exposure.

2. MATERIALS AND METHODS

2.2 Subjects

The study was based on serum samples of CLZ-treated patients from a routine therapeutic drug monitoring (TDM) service, who had also been subjected to genotyping, at the Center for Psychopharmacology, Diakonhjemmet Hospital (Oslo, Norway), during January 2018 – January 2020.

Information about CLZ dose, concomitant medications and time interval between the last dose and sampling time was retrieved from the TDM requisition forms. Inclusion criteria were *i)* serum samples of CLZ drawn 10-24 hours after the last dose intake, *ii)* information about dosing of CLZ provided on the requisition forms, and *iii)* information about smoking. Samples were excluded if patient age was below 18 or above 75 years, or when review of the requisition forms disclosed concomitant use of CNS drugs interacting with the metabolism of CLZ, i.e. the CYP1A2 inhibitor fluvoxamine, antiepileptic CYP inducers (carbamazepine, phenytoin, and phenobarbital), valproic acid or the UGT1A4 substrate lamotrigine. In addition, samples were excluded if there was discrepancy between the smoking habits given on the requisition forms and cotinine levels (nicotine metabolite) in the accompanying serum samples. Since cotinine is present in small amounts in blood regardless of smoking, a conservative and pragmatic threshold value of cotinine areal-under-curve (AUC) was set to $1,00 \times 10^6$ based on reviewing the cotinine levels in smokers vs. nonsmokers (supplementary table 1). Thus, patients who were smokers or nonsmokers based on the information on the requisition forms, would be excluded if the cotinine levels were below or above this threshold, respectively. The intention of using cotinine in the study was to confirm the information about smoking habits written on the requisition forms. The study included all samples (i.e. multiple samples per patients) obtaining the abovementioned criteria.

The study was approved by the Regional Committee for Medical and Health Research Ethics (2014/1185) and did not require informed patient consent as only historical data were applied without the potential to cause any harm.

2.2 Analyses of Clozapine, Clozapine N-oxide, N-desmethylclozapine, Clozapine 5N/N⁺-glucuronide and Cotinine serum concentrations

During the time course of retrospective data collection, an ultra-high-performance LC (UHPLC)-high resolution mass spectrometry (HRMS) method was applied for identification and quantification of CLZ, CLZ *N*-oxide, *N*-DMC, CLZ $5N/N^+$-glucuronide and cotinine. Briefly, the serum samples were prepared by protein precipitation in 96-deep well plates using a Microlab Star pipetting robot (Hamilton, Reno, NV, USA) in a semi-automated sample

preparation procedure. The LC system was a Vanquish-UHPLC (Thermo Fisher Scientific, Waltham, MA, USA), and chromatographic separation was performed by an XBridge BEH C18-column (2.6 µm, 2.1x75 mm; Waters, Milford, MA, USA) using gradient elution at 35 °C with a mix of ammonium acetate buffer (pH = 4.8) and acetonitrile (20-52%). The retention times were 0.4, 0.8, 1.3, 1.5, 1.6, and 1.7 min for cotinine, CLZ 5N-glucuronide, CLZ N^+-glucuronide, N-DMC, CLZ N-oxide and CLZ, respectively. Detection used a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), operated in positive ionisation mode acquiring full scan data at a resolution of 70,000 within the 100-1,500 Da scan range. The compounds were quantified in full scan acquisition mode, while accurate data dependent MS2 analysis was simultaneously triggered to permit confirmation of their identification. The method for identification and quantification of CLZ, N-DMC and CLZ N-oxide are validated and used in routine analyses at Diakonhjemmet Hospital. CLZ and 13C-d3-CLZ (internal standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Alsachim (Illkirch-Graffenstaden, France), respectively.

For CLZ 5N- and N^+-glucuronide and cotinine, their levels in the serum samples were assessed by retrospective reprocessing of the HRMS data file, as described elsewhere for other compounds [20], using the TraceFinder 4.1 software (Thermo Fisher Scientific, Waltham, MA, USA). The CLZ $5N/N^+$-glucuronides and cotinine were tentatively identified by accurate mass (the protonated molecular ion was present within a mass tolerance of 5 ppm), isotope ratio and interpretation of the MS2 spectrum. Identification of CLZ 5N-glucuronide and CLZ N^+-glucuronide were confirmed using retention time and matched MS2 spectrum by analysing reference standards purchased commercially (TLC Pharmaceutical Standards, Newmarket, ON, Canada). Thus, as the extracted-ion chromatogram for the exact mass for CLZ N-glucuronide revealed two peaks, where the two peaks were confirmed to be CLZ 5N-glucuronide and CLZ N^+-glucuronide, respectively, by analyses of reference standards.

2.3 UGT1A4*3 and UGT2B (rs1670747G>A and rs2926038G>C) Genotyping

Genomic DNA was extracted from blood samples using a MagNA Pure 24 Instrument with MagNA Pure 24 Total NA Isolation kit (Roche Diagnostics, Germany).

Due to high levels of DNA sequence homology between the *UGT* genes, a Real-Time (RT) PCR method for the *UGT1A4*3* variant (142T>G, *rs2011425*) has not yet been available. Therefore, a novel RT-PCR method was developed at Center for Psychopharmacology using a mixture of allele-specific primers and allele-specific TaqMan probes. The RT-PCR was designed with a common reverse primer (ACC CTT GAG TGT AGC CCA

GC) and two forward primers specific to the *1*-allele (TCA GCA TGC GGG AGG CC**T**) and the *3*-allele (TCA GCA TGC GGG AGG CC**G**) respectively, in addition to allele specific TaqMan probes (VIC – CAT GGA GCT CCC GCA **A**G - *MGB* and 6-FAM – CAT GGA GCT CCC GCA **C**G - *MGB*). The allele specific nucleotides are marked in boldface. The RT-PCR assay was performed by QuantStudio 12K Flex RT-PCR Instrument with QS12K Software, Version 1.2.2 (Thermo Fisher Scientific, Waltham, USA) using 384-well PCR plates. The wells contained 8 µL of PCR mix, which included 1X TaqMan Universal Genotyping Master Mix (Thermo Fisher), 250 nM of the *1*-allele-specific forward primer, 150 nM of the *3*-allele-specific forward primer, 400 nM of the common reverse primer, 200 nM of the *1*-specific VIC-labelled probe, 120 nM of the *3*-specific FAM-labelled probe and around 30 ng of genomic DNA. Samples were heated 95°C for 10 min to initiate Taq DNA polymerase, followed by 50 cycles of amplification (95°C for 15 s and 60°C for 60 s). The RT-PCR method was validated by comparing 92 unique samples with the established restriction fragment length polymorphism (PCR-RFLP) method, described elsewhere [21]. All sample results gave 100% compliance between the methods.

TaqMan assays for *rs1670747G>A* and *rs2926038G>C* genotyping were not available at Thermo Fisher Scientific, therefore assays for two SNPs that were in complete linkage disequilibrium with *rs1670747* and *rs2926038* were purchased (R^2=1 in Europeans) [22] i.e. *rs1670747/**rs416593*** (cat. nr: C_771051_10) and *rs2926038/**rs1513559*** (Cat nr: C_1509939_10). The RT-PCRs for *rs1670747/**rs416593*** and *rs2926038/**rs1513559*** genotyping were performed as recommended by Thermo Fisher Scientific in 8 µL volumes and same temperature cycles as for *UGT1A4*3*.

Combined haplotypes (UGT2B) from *rs1513559* and *rs416593* were generated using PHASE 2.0 [23]. These two SNPs are located closely (~41 kbp; chr4) and are correlated in a European population (R^2=0.31) [22].

2.4 Statistical Analysis

To evaluate the effect of *UGT1A4*3* allele and *UGT2B* haplotype variants on dose-adjusted serum concentration of CLZ 5*N*-glucuronide, CLZ *N$^+$*-glucuronide *and* the various metabolite-to-parent (MPRs) ratios, linear mixed model analyses were used to allow multiple samples per patient. In these analyses age, sex, blood sampling time and smoking habits were included as covariates. The drug exposure was defined as the dose-adjusted serum concentration (concentration/dose ratio, CD ratio). We used ln-transformed values to ensure normal distributions. Undetectable levels of CLZ glucuronides were truncated to the lowest observed value within each CLZ glucuronide respectively to ensure proper calculation of MPRs. All statistical analyses were performed in SPSS®,

version 21.0 (IBM® SPSS® Statistics, Armonk, NY, USA). GraphPad version 4 was used for graphical presentations (GraphPad Software, San Diego, CA).

3. RESULTS

In total, 79 patients with 216 serum concentrations were included of which 61% of the patients were smokers from information on the requisition forms, which was confirmed by measuring high levels of cotinine (table 1). The age of the population was 43.3 years (SD: 14.7y) comprising of a male majority (62%). In the population, three different *UGT2B* haplotypes were identified. The haplotype frequencies were 10.1%, 11.4% and 78.5% for the *UGT2B:AA*, *UGT2B:GA* and *UGT2B:AT* haplotypes, respectively (*UGT2B:AA: rs1513559A>G* and *rs416593T>A; UGT2B:GA*: *rs1513559A>G* and *rs416593T>A; UGT2B:AT rs1513559A>G* and *rs416593T>A*). In the population, 7.6% were carriers of the *UGT1A4*3* allele, and 13.9% and 3.8% were carriers of one and two alleles of the *UGT2B:GA* haplotype variant, respectively. No significant differences in the Hardy-Weinberg equilibrium were observed between the investigated SNPs (p>0.05). In the population, the average prescribed CLZ dose was 416 mg (table 1). The average absolute serum concentrations of CLZ, *N*-DMC, CLZ *N*-oxide, CLZ 5*N*-glucuronide and CLZ N^+-glucuronide were 1496, 1011, 131, 83 and 71 nM, respectively. Smokers had an average cotinine levels 3000-fold higher compared to nonsmokers (table 1 and supplementary figure 1).

In the multivariate analyses adjusting for smoking habits, sex, age and sampling time, *UGT1A4*3* carriers were observed with a 1.5-fold higher CD ratio of CLZ compared to noncarriers, but the difference was not statistically significant (*UGT1A4*3* carriers vs noncarriers: 5.46 vs 3.69 nM/mg, p=0.064, table 2 and supplementary table 1). Furthermore, *UGT2B:GA/GA* and *UGT2B:GA/AT* carriers were associated with decreased CD ratios of *N*-DMC (*UGT2B:GA/GA vs UGT2B:AT/AT:* 1.44 vs 2.75 nM/mg, p=0.019; *UGT2B:GA/AT* vs *UGT2B:AT/AT*: 2.08 vs 2.75 nM/mg, p=0.062; table 2 and supplementary table 1) as compared to the *UGT2B:AT/AT* carriers. Compared to *UGT2B:AT/AT* carriers, significantly reduced *N*-DMC-to-CLZ MPR ratios were observed in *UGT2B:GA/GA* and *UGT2B:GA/AT* carriers, respectively (*UGT2B:GA/GA vs UGT2B:AT/AT*: 0.42 vs 0.69, p=0.012; *UGT2B:GA/AT* vs *UGT2B:AT/AT*: 0.58 vs 0.69 nM/mg, p=0.106; table 2). The CD ratio of CLZ 5*N*-glucuronide was reduced by 75% in carriers of *UGT2B:GA/GA* haplotype vs *UGT2B:AT/AT* carriers (*UGT2B:GA/GA vs UGT2B:AT/AT*: 0.038 vs 0.16 nM/mg, p=0.030; table 2). Accordingly, the CLZ 5*N*-glucuronide-to-CLZ MPR ratio was reduced by the same degree in *UGT2B:GA/GA* carriers (*UGT2B:GA/GA vs UGT2B:AT/AT*: 0.011 vs 0.039, p=0.030; table 2) when comparing to noncarriers. Compared to noncarriers, *UGT1A4*3* carriers had a nominally increased CD ratio of CLZ 5*N*-glucuronide by 100%, but this effect was not significant (p=0.14, table 2 and supplementary table 1). Compared to *UGT2B:AT/AT* carriers, *UGT2B:GA/GA* and *UGT2B:GA/AT* carriers had 70% and 50% (*UGT2B:GA/GA vs UGT2B:AT/AT*: 0.045 vs 0.15 nM/mg, p=0.021; *UGT2B:GA/AT vs UGT2B:AT/AT*: 0.076 vs 0.15 nM/mg, p=0.016; table 2 and supplementary table 1) reduced CD

ratios of CLZ N^+-glucuronide levels, respectively. Accordingly, the CLZ N^+-glucuronide-to-CLZ MPR ratios were reduced in *UGT2B:GA/GA* and *UGT2B:GA/AT* carriers vs *UGT2B:AT/AT* carriers (*UGT2B:GA/GA vs UGT2B:AT/AT*: 0.012 vs 0.039 nM/mg, p=0.022; *UGT2B:GA/AT vs UGT2B:AT/AT*: 0.021 vs 0.039 nM/mg, p=0.028; table 2 and supplementary table 1).

In the mixed model, smoking reduced the CD ratio of CLZ by 35% at average (p<0.001; supplementary table 1). No effects on the various MPRs and CD ratios of CLZ and CLZ metabolites/glucuronides were observed in *UGT2B:AA* haplotype vs. *UGT2B:AT/AT* carriers (p>0.05; figure 1, table 2 and supplementary table 1). No effect on CLZ N-oxide was observed among the various *UGT1A4*3* and *UGT2B:GA* carriers vs. noncarriers (figure 1C).

4. DISCUSSION

In the present study, the carriers of the *UGT2B:GA* haplotype determined the variants (*rs1513559* and *rs416593*) had significantly decreased CLZ glucuronidation. Thus, the study confirms the importance of these variants for CLZ metabolism and glucuronidation as identified by the recent GWASs [10, 11]. No significant impact on CLZ exposure was observed in carriers of the *UGT2B:GA* haplotype nor the *UGT1A4*3* allele variant when compared to noncarriers. However, the study demonstrated a potential increased CLZ 5*N*-glucuronidation phenotype of the *UGT1A4*3* allele variant as previously shown *in vitro* [19]. The altered CLZ glucuronidation by the *UGT2B:GA* haplotype and *UGT1A4*3* allele variants should be further investigated in future clinical studies to assess them in relation to CLZ tolerability.

The reduced glucuronidation rate of CLZ observed in *UGT2B:GA/GA* carriers did not affect the serum levels of CLZ, and thus the clinical effect is unlikely to be changed. However, altered glucuronidation by the *UGT2B:GA* haplotype and *UGT1A4*3* allele variants may involve a shift in other metabolic pathways relevant for the tolerability of CLZ. One of the suggested toxic metabolites, i.e. the CLZ nitrenium ion, is shown to be involved in CLZ-induced agranulocytosis [24]. Therefore, any alterations of the CLZ metabolic pathway caused by changed UGT-mediated glucuronidation may be of clinical relevance in CLZ treatment. Recently, a positive association was shown between levels of *N*-DMC and neutrophil granulocyte [25], which potentially indicate that a shift in metabolic pathways may have an impact on CLZ tolerability. Therefore, one might hypothesize that carriers of the *UGT2B:GA/GA* diplotype may have increased generation of potential toxic metabolites such as the CLZ nitrenium ion, since they show reduced glucuronidation-mediated trapping of the reactive CLZ metabolite. In fact, carriers of *UGT2B:GA/GA* had decreased *N*-DMC levels and *N*-DMC-to-CLZ MPR ratio, further supporting this hypothesis of a nonprotective role of the *GA* haplotype in clozapine tolerability. The effect size on *N*-DMC-to-CLZ MPR ratio of the *UGT2B:GA* haplotype was approximately the same as previously reported in a larger study [10]. On the other hand, *UGT1A4*3* carriers seem to have an increased glucuronidation phenotype which may in turn protect against accumulation of CLZ nitrenium ions and may have a favourable clozapine tolerability profile.

The potential increased glucuronidation rate in *UGT1A4*3* carriers has previously been observed in olanzapine-treated patients, an antipsychotic drug with similarities in the chemical structure of clozapine [18]. Thus, the increased function of the *UGT1A4*3* variant allele on glucuronidation of clozapine and olanzapine seems to be analogous. In another study, however, the *UGT1A4*3* variant allele was shown to decrease the exposure of olanzapine by approximately 25%, which was like the effect size of male sex and smoking habits in schizophrenic patients [21]. This was not the case in our cohort, since the *UGT1A4*3* allele variant seems to be associated with

increased CLZ levels, although the number of *UGT1A4*3* carriers is low (n=6). Thus, it is important to evaluate this observation in larger studies.

Many of the limitations of the present study are due to the naturalistic setting of the data collection. The study lacked information about comorbidity, organ functions, body weight and potential use of interacting drugs, except from fluvoxamine, carbamazepine, phenytoin, phenobarbital, lamotrigine and valproic acid, which are regularly written on the requisition forms. On the other hand, we had access to several factors that could affect the glucuronidation rate of CLZ, including sex, age and smoking habits, as confirmed by measuring high or low cotinine levels in the blood samples, which were adjusted for in the statistical analyses. As the various *UGT* haplotypes are relevant for CLZ pharmacokinetics, they may potentially also be relevant for CLZ tolerability. However, the number of patients in present study was low and larger clinical studies are warranted, to determine the importance of the *UGT1A4*3* allele and *UGT:GA* haplotype variants in CLZ tolerability.

CONCLUSION

In conclusion, the present study shows that the *UGT2B:GA* carriers have reduced CLZ glucuronidation and the *UGT1A4*3* carriers have potentially increased CLZ 5*N*-glucuronidation. As reactive metabolites play a role in CLZ-induced toxicity, altered glucuronidation in *UGT2B:GA/GA* carriers and *UGT1A4*3* carriers may potentially alter the inactivation of reactive CLZ metabolites, and hence be relevant for the treatment tolerability.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Regional Committee for Medical and Health Research Ethics (2014/1185), and did not require informed patient consent as only historical data were applied without the potential to cause any harm.

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the basis of this research. All human procedures followed were in accordance with the Helsinki Declaration of 1975.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

REFERENCES

1.   Leucht, S.; Cipriani, A.; Spineli, L.; Mavridis, D.; Orey, D.; Richter, F.; Samara, M.; Barbui, C.; Engel, R. R.; Geddes, J. R.; Kissling, W.; Stapf, M. P.; Lassig, B.; Salanti, G.; Davis, J. M. Comparative efficacy and tolerability of 15 antipsychotic drugs in schizophrenia: a multiple-treatments meta-analysis. *Lancet,* **2013**, *382*(9896), 951-962.

2.   Gerson, S. L.; Meltzer, H. Mechanisms of clozapine-induced agranulocytosis. *Drug safety,* **1992**, *7 Suppl 1*, 17-25.

3.   Wong, J.; Delva, N. Clozapine-induced seizures: recognition and treatment. *Can. J. Psychiatry,* **2007**, *52*(7), 457-463.

4.   Joober, R.; Boksa, P. Clozapine: a distinct, poorly understood and under-used molecule. *J. Psychiatry. Neurosci.,* **2010**, *35*(3), 147-149.

5.   Hiemke, C.; Bergemann, N.; Clement, H. W.; Conca, A.; Deckert, J.; Domschke, K.; Eckermann, G.; Egberts, K.; Gerlach, M.; Greiner, C.; Grunder, G.; Haen, E.; Havemann-Reinecke, U.; Hefner, G.; Helmer, R.; Janssen, G.; Jaquenoud, E.; Laux, G.; Messer, T.; Mossner, R.; Muller, M. J.; Paulzen, M.; Pfuhlmann, B.; Riederer, P.; Saria, A.; Schoppek, B.; Schoretsanitis, G.; Schwarz, M.; Gracia, M. S.; Stegmann, B.; Steimer, W.; Stingl, J. C.; Uhr, M.; Ulrich, S.; Unterecker, S.; Waschgler, R.; Zernig, G.; Zurek, G.; Baumann, P. Consensus guidelines for therapeutic drug monitoring in neuropsychopharmacology: update 2017. *Pharmacopsychiatry,* **2018**, *51*(1-02), 9-62.

6.   Dain, J. G.; Nicoletti, J.; Ballard, F. Biotransformation of clozapine in humans. *Drug. Metab. Dispos.,* **1997**, *25*(5), 603-609.

7.   Thorn, C. F.; Muller, D. J.; Altman, R. B.; Klein, T. E. PharmGKB summary: clozapine pathway, pharmacokinetics. *Pharmacogenet. Genomics,* **2018**, *28*(9), 214-222.

8.   van der Weide, J.; Steijns, L. S.; van Weelden, M. J. The effect of smoking and cytochrome P450 CYP1A2 genetic polymorphism on clozapine clearance and dose requirement. *Pharmacogenetics,* **2003**, *13*(3), 169-172.

9.   Villard, P. H.; Herber, R.; Seree, E. M.; Attolini, L.; Magdalou, J.; Lacarelle, B. Effect of cigarette smoke on UDP-glucuronosyltransferase activity and cytochrome P450 content in liver, lung and kidney microsomes in mice. *Pharmacol. Toxicol.,* **1998**, *82*(2), 74-79.

10.   Smith, R. L.; O'Connell, K.; Athanasiu, L.; Djurovic, S.; Kringen, M. K.; Andreassen, O. A.; Molden, E. Identification of a novel polymorphism associated with reduced clozapine concentration in

schizophrenia patients-a genome-wide association study adjusting for smoking habits. *Transl. Psychiatry,* **2020**, *10*(1), 198.

11.   Pardinas, A. F.; Nalmpanti, M.; Pocklington, A. J.; Legge, S. E.; Medway, C.; King, A.; Jansen, J.; Helthuis, M.; Zammit, S.; MacCabe, J.; Owen, M. J.; O'Donovan, M. C.; Walters, J. T. R. Pharmacogenomic Variants and Drug Interactions Identified Through the Genetic Analysis of Clozapine Metabolism. *Am. J. Psychiatry,* **2019**, *176*(6), 477-486.

12.   Genomes Project, C.; Auton, A.; Brooks, L. D.; Durbin, R. M.; Garrison, E. P.; Kang, H. M.; Korbel, J. O.; Marchini, J. L.; McCarthy, S.; McVean, G. A.; Abecasis, G. R. A global reference for human genetic variation. *Nature,* **2015**, *526*(7571), 68-74.

13.   Mijderwijk, H.; Klimek, M.; van Beek, S.; van Schaik, R. H.; Duivenvoorden, H. J.; Stolker, R. J. Implication of UGT2B15 Genotype Polymorphism on Postoperative Anxiety Levels in Patients Receiving Lorazepam Premedication. *Anesth Analg,* **2016**, *123*(5), 1109-1115.

14.   Murphy, S. E.; von Weymarn, L. B.; Parenteau, M.; Stepanov, I.; Tiirikainen, M.; LeMarchand, L.; Park, S. L. Influence of UGT2B10 Genotype on Urinary Excretion of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol-N-glucuronide by African American Smokers. *Chem Res Toxicol,* **2018**, *31*(3), 168-175.

15.   Benoit-Biancamano, M. O.; Adam, J. P.; Bernard, O.; Court, M. H.; Leblanc, M. H.; Caron, P.; Guillemette, C. A pharmacogenetics study of the human glucuronosyltransferase UGT1A4. *Pharmacogenet. Genomics,* **2009**, *19*(12), 945-954.

16.   Ehmer, U.; Vogel, A.; Schutte, J. K.; Krone, B.; Manns, M. P.; Strassburg, C. P. Variation of hepatic glucuronidation: Novel functional polymorphisms of the UDP-glucuronosyltransferase UGT1A4. *Hepatology,* **2004**, *39*(4), 970-977.

17.   Erickson-Ridout, K. K.; Zhu, J.; Lazarus, P. Olanzapine metabolism and the significance of UGT1A448V and UGT2B1067Y variants. *Pharmacogenet. Genomics,* **2011**, *21*(9), 539-551.

18.   Haslemo, T.; Loryan, I.; Ueda, N.; Mannheimer, B.; Bertilsson, L.; Ingelman-Sundberg, M.; Molden, E.; Eliasson, E. UGT1A4*3 encodes significantly increased glucuronidation of olanzapine in patients on maintenance treatment and in recombinant systems. *Clin. Pharmacol. Ther.,* **2012**, *92*(2), 221-227.

19.   Erickson-Ridout, K. K.; Sun, D.; Lazarus, P. Glucuronidation of the second-generation antipsychotic clozapine and its active metabolite N-desmethylclozapine. Potential importance of the UGT1A1 A(TA)(7)TAA and UGT1A4 L48V polymorphisms. *Pharmacogenet. Genomics,* **2012**, *22*(8), 561-576.

20.   Partridge, E.; Trobbiani, S.; Stockham, P.; Charlwood, C.; Kostakis, C. A Case Study Involving U-47700, Diclazepam and Flubromazepam-Application of Retrospective Analysis of HRMS Data. *J. Anal. Toxicol.,* **2018**, *42*(9), 655-660.

21.   Ghotbi, R.; Mannheimer, B.; Aklillu, E.; Suda, A.; Bertilsson, L.; Eliasson, E.; Osby, U. Carriers of the UGT1A4 142T>G gene variant are predisposed to reduced olanzapine exposure--an impact similar to male gender or smoking in schizophrenic patients. *Eur. J. Clin. Pharmacol.,* **2010**, *66*(5), 465-474.

22.   Machiela, M. J.; Chanock, S. J. LDlink: a web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. *Bioinformatics,* **2015**, *31*(21), 3555-3557.

23.   Stephens, M.; Smith, N. J.; Donnelly, P. A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet.,* **2001**, *68*(4), 978-989.

24.   Ng, W.; Kennar, R.; Uetrecht, J. Effect of clozapine and olanzapine on neutrophil kinetics: implications for drug-induced agranulocytosis. *Chem. Res. Toxicol.,* **2014**, *27*(7), 1104-1108.

25.   Smith, R. L.; Kylleso, L.; Haslemo, T.; Andreassen, O. A.; Molden, E. Reduction in N-Desmethylclozapine Level Is Determined by Daily Dose But Not Serum Concentration of Valproic Acid - Indications of a Presystemic Interaction Mechanism. *Ther. Drug. Monit.,* **2019**, *41*(4), 503-508.

FIGURE LEGENDS

Figure **1** Effects of the *UGT1A4*3 Allele*, the *UGT2B:GA* and *UGT2B:AA* Haplotypes on Exposure of Clozapine (CLZ), *N*-desmethylclozapine, CLZ 5*N*-glucuronide, CLZ N^+-glucuronide, and CLZ *N*-oxide. The univariate comparisons of the *UGT1A4*3* allele and the *UGT2B:GA* and *UGT2B:AA* haplotypes on dose-adjusted serum concentrations (CD) of clozapine (A; CLZ), *N*-desmethylclozapine (*N*-DMC) (B), CLZ 5*N*-glucuronide (C), CLZ N^+-glucuronide (D) and CLZ *N*-oxide (E). The horizontal lines represent the mean of the presented subpopulations. Each dot represents a patient sample (both black and grey). Black dots represent the reference group used for statistical comparisons within each allele variant. All values are ln-transformed to ensure normal distribution. Linear mixed model analyses were used and statistical significance was considered at p-value below 0.05.

Supplementary figure **1** The serum cotinine levels in nonsmokers and smokers. Information about smoking habits (non-smoker or smoker) were retrieved from the TDM requisition forms. The dotted horizontal line represents the threshold of exclusion of cotinine samples above for non-smoker and cotinine levels below for smokers, respectively.

TABLES

Table **1** Population Characteristics.

Variables	Values
Baseline characteristics:	
Female/Male, n (samples)	30 (66) /49 (150)
Age, mean years (SD)	43.3 (14.7)
Sampling time, mean hrs (SD)	14.0 (2.59)
Dose, mg (SD)	416 (193)
Confirmed smokers (%)	48 (61%)
Cotinine levels in smokers/non-smoker, AUC (SD)	333×10^6 (215)/ 0.11×10^6 (0.125)
Genotype characteristics:	
UGT1A4 allele carriers:	
*1/*3*, n (samples)	6 (18)
*3/*3*, n (samples)	0 (0)
UGT2B haplotype carriers:	
UGT2B:AT/AT, n (samples)	49 (122)
UGT2B:AT/AA, n (samples)	15 (57)
UGT2B:GA/AT, n (samples)	11 (30)
UGT2B:GA/AA, n (samples)	1 (1)
UGT2B:GA/GA, n (samples)	3 (6)
Absolute concentrations:	
CLZ, nM mean (SD)	1496 (920)
N-DMC, nM mean (SD)	1011 (583)
CLZ 5*N*-glucuronide, nM mean (SD)	83 (99)
CLZ N^+- glucuronide, nM mean (SD)	71 (88)
CLZ *N*-oxide, nM mean (SD)	131 (84)

CLZ, clozapine; *N*-DMC, *N*-desmethylclozapine; P, p-value; SD, Standard deviation; SNP, single nucleotide polymorphism.
UGT2B haplotypes are defined based on SNPs *rs1513559A>G* and *rs416593T>A*

Table **2** The Effect of *UGT1A4*3* Allele and *UGT2B* Haplotype Variants on Exposure and Metabolic Ratios of Clozapine, *N*-desmethylclozapine, CLZ 5*N*-glucuronide, and CLZ N^+-glucuronide. The effect of *UGT1A4 *3* allele and *UGT2B* haplotype (combined *rs1513559A>G* and *rs416593T>A*) variants on dose-adjusted serum concentrations (CD) of clozapine (CLZ), *N*-desmethylclozapine (*N*-DMC), clozapine glucuronides and various metabolic ratios adjusted for smoking habits, sex, age and sampling withdrawal time.

	MPR *N*-DMC Fold change (95% CI)	P	MPR CLZ 5*N*-gluc. Fold change (95% CI)	P	MPR CLZ N^+-gluc. Fold change (95% CI)	P	CD CLZ N^+-gluc Fold change (95% CI)	P	CD *N*-DMC Fold change (95% CI)	P	CD CLZ Fold change (95% CI)	P	CD CLZ 5*N*-gluc Fold change (95% CI)	P
UGT2B haplotype:														
AT/AT (ref)	-		-		-		-		-		-		-	
GA/AT	0.84 (0.68, 1.0)	0.11	1.4 (0.72, 2.6)	0.34	0.55 (0.32, 0.94)	0.028	0.50 (0.28, 0.88)	0.016	0.76 (0.57, 1.0)	0.062	0.90 (0.65, 1.3)	0.54	1.2 (0.58, 2.6)	0.58
GA/GA	0.61 (0.42, 0.90)	0.012	0.28 (0.087, 0.88)	0.030	0.32 (0.12, 0.84)	0.022	0.29 (0.10, 0.83)	0.021	0.53 (0.31, 0.90)	0.019	0.86 (0.47, 1.6)	0.63	0.24 (0.062, 0.94)	0.041
AA/AT	0.96 (0.79, 1.2)	0.63	0.81 (0.46, 1.4)	0.45	0.82 (0.52, 1.3)	0.42	0.85 (0.52, 1.4)	0.51	1.0 (0.77, 1.3)	0.97	1.0 (0.78, 1.4)	0.78	0.84 (0.44, 1.6)	0.59
GA/AA	0.61 (0.31, 1.2)	0.15	0.59 (0.074, 4.8)	0.62	0.22 (0.037, 1.3)	0.092	0.51 (0.075, 3.5)	0.49	1.4 (0.55, 3.6)	0.46	2.3 (0.79, 6.8)	0.12	1.4 (0.12, 16)	0.80
UGT1A4 genotype:														
*3 vs *1/*1*	0.92 (0.71, 1.2)	0.54	1.3 (0.62, 2.9)	0.44	1.0 (0.52, 1.9)	0.99	15 (0.73, 2.9)	0.28	1.4 (0.95, 2.0)	0.094	15 (0.98, 2.2)	0.064	2.0 (0.79, 5.0)	0.14

Linear mixed model analyses were used allowing inclusion of multiple measurements per patients. *UGT2B:AT/AT* genotype was used as reference. The statistical analyses were performed after including age, sex, smoking habits and sampling time as covariates.

UGT2B haplotype is based on the combination of *SNPs rs1513559A>G* and *rs416593T>A*. *UGT2B:**GA**, rs1513559**G** and rs416593**A**; UGT2B:**AA**, rs1513559**A** and rs416593**A**; UGT2B:**AT**, rs1513559**A** and rs416593**T***.

CD, CD ratio (concentration-to-dose ratio); MPR, metabolite-to-CLZ ratio; *N*-DMC, *N*-desmethylclozapine.

SUPPLEMENTARY MATERIAL

Supplementary table **1** The Effect of *UGT1A4*3* Allele, *UGT2B* Haplotype Variants, Age, Sex and Withdrawal Time on Exposure and Metabolic Ratios of Clozapine, *N*-desmethylclozapine, CLZ 5*N*-glucuronide, and CLZ *N*-glucuronide. The table shows the complete statistical model of *UGT1A4*3* allele and *UGT2B* haplotype (combined *rs1513559A>G* and *rs416593T>A*) variants on dose-adjusted serum concentrations of clozapine, *N*-desmethylclozapine (*N*-DMC), clozapine glucuronides and various metabolic ratios adjusted for smoking habits, sex, age and sampling withdrawal time.
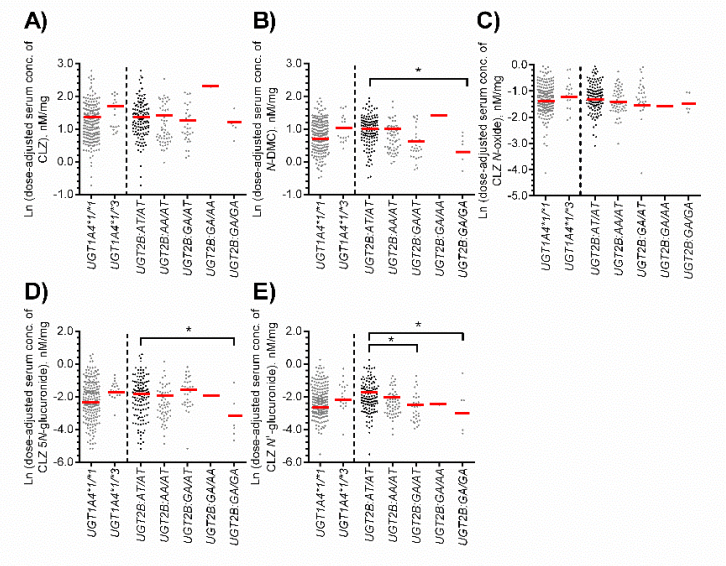
Variables	CD CLZ β value, nM/mg (SE)	P	CD N-DMC β value, nM/mg (SE)	P	CD CLZ 5N-glucuronide β value, nM/mg (SE)	P	CD CLZ N^+-glucuronide β value, nM/mg (SE)	P	MPR N-DMC β value (SE)	P	MPR CLZ 5N-glucuronide β value (SE)	P	MPR CLZ N^+-glucuronide β value (SE)	P
Intercept	1.46 (0.26)	<0.001	0.96 (0.23)	<0.001	-2.40 (0.62)	<0.001	-1.71 (0.49)	0.001	-0.49 (0.17)	0.003	-3.86 (0.52)	<0.001	-3.19 (0.45)	<0.001
UGT1A4*3 carriers	0.39 (0.21)	0.064	0.31 (0.18)	0.094	0.68 (0.46)	0.14	0.37 (0.34)	0.28	-0.082 (0.13)	0.54	0.30 (0.39)	0.44	0.0036 (0.33)	0.99
UGT2B haplotypes:														
AT/AT (ref)	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
AA/AT	0.041 (0.15)	0.78	-0.0047 (0.13)	0.97	-0.18 (0.33)	0.59	-0.16 (0.25)	0.51	-0.046 (0.094)	0.63	-0.21 (0.28)	0.45	-0.19 (0.23)	0.42
GA/AT	-0.10 (0.17)	0.54	-0.28 (0.15)	0.062	0.21 (0.37)	0.58	-0.69 (0.28)	0.016	-0.17 (0.11)	0.11	0.31 (0.32)	0.34	-0.60 (0.89)	0.028
GA/AA	0.84 (0.54)	0.12	0.35 (0.47)	0.46	0.32 (1.24)	0.80	-0.67 (0.97)	0.49	-0.50 (0.34)	0.15	-0.52 (1.05)	0.62	-1.52 (0.87)	0.092
GA/GA	-0.15 (0.30)	0.63	-0.64 (0.27)	0.019	-1.42 (0.68)	0.041	-1.23 (0.52)	0.021	-0.49 (0.19)	0.012	-1.28 (0.58)	0.030	-1.15 (0.49)	0.022
Smoker	-0.44 (0.12)	<0.001	-0.29 (0.10)	0.007	-0.36 (0.26)	0.18	-0.15 (0.20)	0.46	0.15 (0.075)	0.052	0.085 (0.22)	0.71	0.29 (0.19)	0.13
Male sex	0.016	0.90	-0.018 (0.11)	0.87	0.32 (0.28)	0.26	-0.25 (0.21)	0.23	-0.034 (0.078)	0.66	0.30 (0.23)	0.21	-0.28 (0.20)	0.16
Age (per year)	0.001 (0.004)	0.73	0.001 (0.003)	0.72	0.011 (0.0084)	0.193	0.0029 (0.006)	0.65	-0.002 (0.002)	0.94	0.010 (0.007)	0.16	0.002 (0.006)	0.70
Blood sampling (per hr)	-0.0043 (0.0095)	0.65	0.0029 (0.008)	0.72	-0.020 (0.024)	0.419	-0.015 (0.021)	0.47	0.0076 (0.0056)	0.178	-0.016 (0.021)	0.44	-0.011 (0.018)	0.54

All CD values are presented as Ln-transformed values to ensure normal distribution. Linear mixed model analyses were used to include multiple measurements per patients. *UGT2B AT/AT* genotype was used as reference. The statistical analyses were performed after including age, sex, smoking habits and sampling time as covariates.
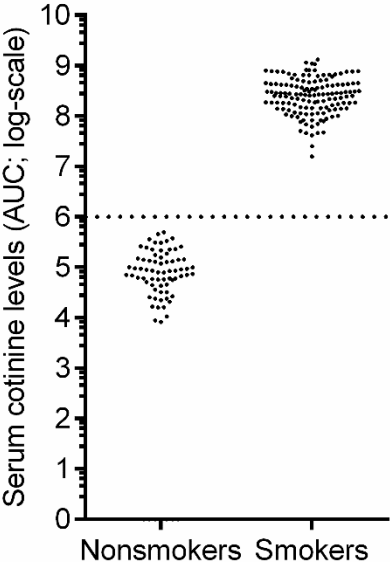*UGT2B* haplotype is based on the combination of *SNPs rs1513559A>G* and *rs416593T>A*. *UGT2B:GA*, *rs1513559G* and *rs416593A*; *UGT2B:AA*, *rs1513559A* and *rs416593A*; *UGT2B:AT*, *rs1513559A* and *rs416593T*.
CD, CD ratio (concentration-to-dose ratio); MPR, metabolite-to-CLZ ratio; SE, standard error; SNP, single nucleotide polymorphism; *N*-DMC, *N*-desmethylclozapine.

Figure 1:

Supplementary figure 1: