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ARTICLE

Impact of NFIB and CYP1A variants on clozapine serum concentration—A retrospective naturalistic cohort study on 526 patients with known smoking habits

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Abstract

Clinical response of clozapine is closely associated with serum concentration. Although tobacco smoking is the key environmental factor underlying interindividual variability in clozapine metabolism, recent genome-wide studies suggest that *CYP1A* and *NFIB* genetic variants may also be of significant importance, but their quantitative impact is unclear. We investigated the effects of the *rs2472297 C>T* (*CYP1A*) and *rs28379954 T>C* (*NFIB*) polymorphisms on serum concentrations in smokers and nonsmokers. The study retrospectively included 526 patients with known smoking habits (63.7% smokers) from a therapeutic drug monitoring service in Norway. Clozapine dose-adjusted concentrations (C/D) and patient proportions with subtherapeutic levels (<1070 nmol/L) were compared between *CYP1A*/*NFIB* variant allele carriers and homozygous wild-type carriers (noncarriers), in both smokers and nonsmokers. Clozapine C/D was reduced in patients carrying *CYP1A-T* and *NFIB-C* variants versus noncarriers, both among smokers (−48%; $p < 0.0001$) and nonsmokers (−35%; $p = 0.028$). Patients who smoke carrying *CYP1A-T* and *NFIB-C* variants had a 66% reduction in clozapine C/D versus nonsmoking noncarriers ($p < 0.0001$). The patient proportion with subtherapeutic levels was 2.9-fold higher in patients who smoke carrying *NFIB-C* and *CYP1A-T* variants versus nonsmoking noncarriers ($p < 0.0001$). In conclusion, *CYP1A* and *NFIB* variants have significant and additive impact on clozapine dose requirements for reaching target serum concentrations. Patients who smoke carrying the studied *CYP1A* and *NFIB* variants, comprising 2.5% of the study population, may need threefold higher doses to prevent risk of clozapine undertreatment. The results suggest that pre-emptive genotyping of *NFIB* and *CYP1A* may be utilized to guide clozapine dosing and improve clinical outcomes in patients with treatment-resistant schizophrenia.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Serum concentration of clozapine exhibits extensive interindividual variability during standard dosing. Cigarette smoking is known to be of great importance for the variability in clozapine metabolism, but the quantitative impact of pharmacogenetic variability is unclear.

WHAT QUESTION DID THIS STUDY ADDRESS?

We investigated the quantitative effects of *CYP1A* and *NFIB* variant alleles on clozapine serum levels in a psychiatric patient cohort ($n = 526$) with known smoking habits.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

CYP1A and *NFIB* variants have significant and additive effects on dose-adjusted clozapine serum concentration. Patients carrying the studied *CYP1A* and *NFIB* variants exhibited significant reductions in clozapine serum concentration. The clinical impact of the genotypes is particularly relevant in smokers, where variant allele carriers had an approximately threefold increased risk of subtherapeutic clozapine levels, which is compliant with a 66% lower dose-adjusted clozapine concentration among these patients.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

The study shows that *CYP1A* and *NFIB* variants are important determinants of clozapine serum concentration. Genotyping of *CYP1A* and *NFIB* in addition to information on smoking status may be used as a clinical tool for precision dosing of clozapine to shorten the time to reach effective concentrations and improve outcome in patients with treatment-resistant schizophrenia.

INTRODUCTION

Clozapine exhibits an overall superior efficacy compared with other antipsychotics for schizophrenia treatment,[1,2] and it is the only drug indicated for use in treatment-resistant schizophrenia (TRS).[3] Despite its superiority to other antipsychotics, up to 40% of patients with TRS do not respond satisfactorily to clozapine,[4] which may be due to disease heterogeneity, nonadherence, and subtherapeutic serum concentrations. In addition to the mandatory monitoring of neutrophil count, therapeutic drug monitoring (TDM) of clozapine serum levels is therefore recommended to ensure adequate clozapine dosing and exposure[5,6] before implementing potential augmentation therapy.[7,8]

The therapeutic response to clozapine has been shown to be closely associated with serum concentrations, where 1070 nmol/L (350 ng/ml) is defined as the general lower threshold to obtain optimal clinical response.[9–11] However, clozapine serum levels exhibit extensive interindividual variability and, consequently, the patients may experience different treatment outcomes at similar dosing. It has been shown that sex, age, and smoking habits explain only about 50% of variability in plasma clozapine levels,[12,13] with tobacco smoking being the key environmental factor that leads to 30%–40% reduction in clozapine serum levels[14] by inducing the expression of CYP1A human isoforms and UDP-glucuronosyltransferases.[15,16] However, clozapine is subjected to complex hepatic metabolism, where CYP1A2 and CYP3A4 are the main enzymes responsible for demethylation of clozapine into its major metabolite *N*-desmethylclozapine, which may mediate both favorable and unfavorable effects.[17]

Although smoking has a large impact on clozapine levels, much of the unexplained pharmacokinetic variability likely occurs due to drug–drug interactions and polymorphisms in genes encoding drug metabolism and transport. In a genome-wide association study (GWAS), Pardiñas et al.[18] demonstrated that minor allele carriers of the *CYP1A rs2472297 C>T* (*CYP1A-T*) polymorphism exhibited significantly lower clozapine plasma concentrations. This polymorphism is located at an intergenic region between *CYP1A1* and *CYP1A2*, in which many regulatory elements, called xenobiotic response elements, reside.[19] The minor allele variant *CYP1A-T* has previously been associated to increased metabolism of the CYP1A2 substrates olanzapine[20] and caffeine,[21] and increased coffee consumption[22] supporting the significant association with clozapine serum levels reported by Pardiñas et al.[18] who did not have access to smoking habits.

Recently, in another GWAS in patients with known smoking habits, we identified a novel variant in the gene encoding the nuclear factor I B (NFIB), which was also significantly associated with reduced clozapine concentrations, but only when adjusting for smoking habits as a covariate.[23] NFIB is involved in embryonic development of various organ systems,[24,25] but it has not before been described to be involved in the regulation of drug-metabolizing enzymes. However, in heterozygous carriers of the *NFIB rs28379954 C* (*NFIB-C*) variant, comprising about 5% in White populations, an almost 40% lower clozapine dose-adjusted (C/D) serum concentration of clozapine was found.[23] Moreover, smokers who were also minor allele carriers exhibited significantly higher risk of subtherapeutic clozapine serum levels compared with homozygous carriers of the major *NFIB-T* allele.[23]

Based on the results of the previous GWASs, we hypothesized that carriers of both the *NFIB-C* and *CYP1A-T* variants comprise a patient subgroup with a particularly high risk of clozapine undertreatment. Therefore, the aim of the current study was to investigate the quantitative effects of the two pharmacogenetic variants on clozapine serum concentration in a Norwegian population of 526 patients with known smoking habits.

METHODS

Study population

The patients were retrospectively included from the TDM/pharmacogenetics service at the Center for Psychopharmacology, Diakonhjemmet Hospital, Oslo, Norway, during January 2005 to January 2021. This service performs analyses of more than 50,000 patient samples each year with certified methods compliant with accreditation standards.

In the present investigation, 80% of the study cohort consisted of patients from our previous GWAS ($n = 420$)[23] in addition to a cohort of newer cases ($n = 106$). The study population included Norwegian inhabitants and comprised mainly White patients (patient ethnicity not confirmed). For patients treated with clozapine in the dose interval 100–1000 mg/day and with available blood samples for genotyping, the inclusion of TDM measurements were performed according to the following criteria: (1) detectable serum concentration of clozapine and *N*-desmethylclozapine, (2) available information on prescribed daily dose and smoking habits indicated on the clozapine TDM requisition form (yes/no), and (3) sampling time since last dose intake within 10–30 h. Although not specifically confirmed, clozapine concentrations were assumed to be at steady-state during blood sampling, as this is clearly stated as a requirement on the TDM requisition forms. Furthermore, by including multiple samples per patient over longer treatment periods, it was ensured that the results reflected steady-state conditions.

Therapeutic drug monitoring measurements were excluded for patients co-medicated with CYP enzyme inducers (phenobarbital, phenytoin, and carbamazepine) or the antidepressant CYP1A2/3A4 inhibitor fluvoxamine. Additionally, measurements with co-medication of valproate were excluded from statistical analysis of *N*-desmethylclozapine-to-clozapine ratio due to its specific interaction with this variable.[26] Patients with inconsistent information about smoking habits for consecutive TDM requests during the time span of the study period were excluded.

The study was approved by the Regional Committee for Medical and Health Research Ethics (#2020/127234) and the Hospital Investigational Review Board. As the study utilized anonymized data and residual blood samples from already performed routine analyses, hence not providing any patient risks or burden, informed consent was not required, however, they were informed about the study and given the opportunity to reserve against being included.

Serum concentration analysis of clozapine and *N*-desmethylclozapine

Two liquid chromatography tandem mass spectrometry (LC–MS/MS) assays were used for serum concentration determination of clozapine and *N*-desmethylclozapine at the Center for Psychopharmacology during the inclusion periods, as previously described in detail.[27] The modification of the analytical assay was due to renewal of analytical instrumentation; however, the methods were essentially the same in terms of sample preparation (protein precipitation), mobile phase composition (gradient elution), and internal standard (promazine), and all modifications were cross-validated according to the criteria for routine TDM.

Concisely, the most recent methods were performed with initial sample purification by protein precipitation; the supernatant was injected into an ultrahigh-performance LC–MS/MS detection. All the calibration curves were linear ($R^2 > 0.99$) in validated ranges: clozapine, 100–3500 nmol/L and *N*-desmethylclozapine, 100–2500 nmol/L. Lower limit of quantification was 20 nmol/L for both clozapine and *N*-desmethylclozapine. Imprecision and inaccuracy parameters of the assays were less than or equal to 5%.

Genetic analyses

Analyses of *NFIB* and *CYP1A* variant alleles were performed using predesigned TaqMan-based real-time polymerase chain reaction (PCR) assays; *NFIB rs28379954 T>C* (C_59359617_10) and *CYP1A rs2472297 C>T* (C__11773054_10; Thermo Fischer Scientific). The PCR reactions were run on a QuantStudio12K Flex Real-Time PCR System (Thermo Fischer Scientific).

Defining patient subgroups

The study population was stratified based on known smoking habits (smokers or nonsmokers) and then into subgroups according to targeted genotyping of the *CYP1A rs2472297 C>T* and *NFIB rs28379954 T>C* polymorphisms. To simplify the data presentation and improve statistical power, heterozygous and homozygous minor allele carriers of the *CYP1A rs2472297 C>T* polymorphism were merged into a single subgroup. The resulting four subgroups were: (1) carriers of both *CYP1A-T* and *NFIB-C* variants, (2) carriers of the *NFIB-C* variant alone, (3) carriers of the *CYP1A-T* variant alone, and (4) homozygous carriers of wild type alleles of both *CYP1A* and *NFIB* (noncarriers, the reference group).

Outcome measures and statistics

The main outcome measure of the study was the quantitative effects of *NFIB-C* and *CYP1A-T* variants on clozapine C/D, absolute serum concentration, and *N*-desmethylclozapine-to-clozapine metabolic ratio in smokers and nonsmokers, respectively. To achieve this, clozapine C/D, absolute concentration, and metabolic ratio estimates of minor allele carriers (subgroups 1–3) were compared with the reference subgroup. Clozapine C/D (nmol/L/mg/day) is a pharmacokinetic measure reflecting the rate of overall clozapine metabolism via a range of different pathways/enzymes and was simply calculated as a ratio of absolute concentration (nmol/L) and total daily dose of clozapine (mg/day). Absolute clozapine serum concentration reflects the actual exposure for mediating the clinical effect. As measurements of the main metabolite were available, we calculated the metabolic ratio of *N*-desmethylclozapine-to-clozapine serum concentrations which indicates the rate of metabolism via that specific pathway.

The patient proportions with subtherapeutic levels of clozapine were compared between variant allele carriers and homozygous wild type allele carriers of *CYP1A* and *NFIB* as a secondary outcome measure. Most of the patients had multiple TDM measurements of clozapine during the study period and they were classified as having subtherapeutic clozapine serum levels at median absolute clozapine serum concentration measurements were below than the lower therapeutic threshold (1070 nmol/L [350 ng/ml]) recommended by Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie (AGNP) guidelines.[6]

To utilize all clozapine TDM measurements included during the study period, we used random intercept multivariate linear mixed model analysis with restricted maximum likelihood for interaction analysis, estimation, and statistical comparisons. Outcome variables in all mixed models were transformed to natural logarithmic (ln) scale. To investigate potential interaction between *CYP1A-T* and *NFIB-C*, we used standalone genotypes, their interaction term, age, gender, smoking habits, and withdrawal time as fixed effects variables. For absolute clozapine serum concentration analysis, total daily prescribed dose was also included as a covariate. Further, we analyzed interaction of genotypes of *CYP1A-T* and *NFIB-C* and smoking habits by adjusting the model for the same covariates and random effects variable. For estimated means and statistical comparisons of genotypes, we used similar models described above, however, we excluded the interaction terms and further stratified the patients according to their smoking habits. Estimated marginal means, and 95% confidence interval (CI) were transformed back to linear scale for representation of the results. Dunnett's method was used to adjust *p* values for multiple comparisons. Proportions of patients with median clozapine serum levels below the target therapeutic range were compared between the genotype subgroups using the Fisher's exact test with Bonferroni correction.

All statistical analysis were performed using R version 4.1.2. For linear mixed model analysis R package lme4 version 1.1.27.1 and lmerTest version 3.1.3 was used. For estimation of marginal means from mixed models and group comparisons, R package emmeans version 1.7.2 was used. Linkage equilibrium analysis was performed using the LDproxy module on the LDlink platform.[28] A *p* value of <0.05 was defined as statistical significance.

RESULTS

Population characteristics

In total, 526 patients with 7910 clozapine TDM measurements were included in the statistical analysis according to the predefined criteria (335 smokers and 191 nonsmokers). An overview of demographic characteristics and general TDM statistics in the study population across genotype subgroups for smokers and nonsmokers, respectively, are

highlighted in Table 1. There were no significant differences in the prescribed daily doses of clozapine between the genotype subgroups among smokers or nonsmokers (Table 1).

Effect of *CYP1A* and *NFIB* variants on clozapine serum levels and metabolic ratio

Comparisons of clozapine absolute serum concentration, C/D, and *N*-desmethylclozapine-to-clozapine metabolic ratio estimates between carriers and noncarriers of *CYP1A*/*NFIB* variant alleles are presented in Table 2 and illustrated in Figures 1, 2, and S1, whereas individual effects of *NFIB* and *CYP1A* genotypes are presented in Table S1.

Assessment of individual quantitative effects of *CYP1A* and *NFIB* polymorphisms in relation to smoking habits revealed similar reductions in clozapine C/D compared with Pardiñas et al.'s[18] and our previous GWASs.[23] Carriers of the *CYP1A-T* variant exhibited significant reductions in clozapine C/D among both smokers (fold-change: 0.82; 95% CI: 0.74–0.92; $p = 0.001$; Table S1) and nonsmokers (fold-change: 0.85; 95% CI: 0.74–0.98; $p = 0.028$; Table S1) relative to patients of *CYP1A C/C* carriers. Furthermore, patients who smoke carrying the *NFIB-C* variant had 26% lower clozapine C/D (fold-change: 0.74; 95% CI: 0.62–0.88; $p = 0.001$; Table S1) compared with *NFIB T/T* carriers, whereas we observed a nonsignificant decline of 18% among nonsmokers ($p = 0.10$; Table S1). Mixed model interaction analyses revealed that there were no significant interaction effects between *CYP1A-T* and *NFIB-C* and smoking status on prediction of clozapine C/D or metabolic ratio (Tables S2 and S3). Accordingly, we observed additive effects on the reduction in clozapine serum concentration when the two variant alleles were inherited together, with the size being approximately similar to the sum of the effects of the individual variant genotypes on C/D and absolute serum concentrations (Tables 2 and S1).

Among smokers, clozapine C/D was 48% lower in patients carrying both *CYP1A-T* and *NFIB-C* variant alleles compared with noncarriers (fold-change: 0.52; 95% CI: 0.39–0.68; $p < 0.0001$; Table 2; Figure 2). In the nonsmoker cohort, we observed a 35% reduction in clozapine C/D (fold-change: 0.65; 95% CI: 0.44–0.95; $p = 0.028$; Table 2 and Figure 2) in carriers both *CYP1A-T* and *NFIB-C* variant alleles compared with noncarriers. Importantly, considering the subgroups at the extremes of clozapine serum levels (i.e., patients who smoke and are also carriers of both *CYP1A-T* and *NFIB-C* variant alleles vs. nonsmoker noncarriers), clozapine C/D was 66% lower in the former subgroup ($p < 0.0001$).

We observed a 19% increase in *N*-desmethylclozapine-to-clozapine metabolic ratio in

TABLE 1 Population characteristics of patients included in the study according to *CYP1A rs2472297 C>T* and *NFIB rs28379954 T>C* genotypes and smoking habits

Variables	Overall	Smokers, *N* = 335 (4793)				Nonsmokers, *N* = 191 (3117)			
		CYP1A C/T - NFIB T/C	*CYP1A C/C - NFIB T/C*	*CYP1A C/T - NFIB T/T*	*CYP1A C/C - NFIB T/T*	*CYP1A C/T - NFIB T/C*	*CYP1A C/C - NFIB T/C*	*CYP1A C/T - NFIB T/T*	*CYP1A C/C - NFIB T/T*
Number of patients, *n* (measurements)	526 (7910)	13 (185)	24 (232)	119 (1688)	179 (2688)	7 (124)	12 (126)	74 (1144)	98 (1723)
Female, *n* (%)	204 (39)	4 (31)	12 (50)*	42 (35)	49 (27)	3 (43)	6 (50)	40 (54)	48 (49)
Age, years; mean (95% CI)	37.7 (35.9, 39.6)	38.3 (32.3, 45.4)	39.9 (35.2, 45.2)	38.0 (35.9, 40.2)	35.8 (34.2, 37.5)	37.9 (29.0, 49.7)	41.8 (34.0, 51.3)*	36.4 (33.5, 39.6)	33.5 (31.2, 36.0)
Withdrawal time, *h*; mean (95% CI)	13.5 (13.3, 13.7)	13.3 (12.7, 14.0)	13.6 (13.1, 14.1)	13.4 (13.2, 13.6)	13.5 (13.3, 13.7)	13.7 (12.9, 14.6)	13.8 (13.2, 14.4)	13.5 (13.2, 13.7)	13.3 (13.1, 13.5)
Total prescribed daily dose, mg/day; mean (95% CI)	308 (289, 329)	321 (253, 407)	292 (244, 350)	364 (337, 394)	331 (311, 353)	327 (234, 455)	294 (229, 378)	264 (239, 293)	276 (253, 302)

Note: Withdrawal time, time difference of last dose intake and sampling time. Estimated means of age, withdrawal time, and total prescribed daily dose were derived from linear mixed model analysis.

Abbreviation: CI, confidence interval.

*Significant statistical test result ($p < 0.05$) when comparing with homozygous wild-type allele carriers in each genotype subgroup.

carriers of both variant alleles compared with noncarriers among smokers (fold-change: 1.19; 95% CI: 1.01–1.40; $p = 0.037$; Table 2 and Figure S1), whereas in the nonsmoker cohort, none of the patient subgroups were associated with significant changes in metabolic ratios ($p > 0.21$; Table 2).

Patient proportions with subtherapeutic levels of clozapine

On average, 15 serum concentration measurements per patient were registered in the study period. In the whole study cohort, 52% of patients ($n = 274$) had median absolute serum measurements of clozapine below the 1070 nmol/L (350 ng/ml) lower therapeutic concentration boundary (Table 3), which is a high frequency potentially reflecting the selection of patients with suboptimal clinical response towards TDM. In the smoker cohort, the proportion of patients who exhibited subtherapeutic clozapine serum levels was 1.7-fold higher in carriers of both variant alleles relative to noncarriers (92% vs. 53%; $p = 0.007$; Table 3). Among nonsmokers, we did not observe any significant changes in proportions of patients below the therapeutic threshold in carriers of both the *CYP1A-T* and *NFIB-C* variant alleles relative to noncarriers ($p = 0.22$; Table 3). Moreover, patients who smoke who are also carriers of both the *CYP1A-T* and *NFIB-C* variant alleles had a 2.9-fold higher proportion of subtherapeutic clozapine levels, compared with patients who are nonsmokers and noncarriers ($p < 0.0001$).

Linkage equilibrium analysis of *CYP1A rs2472297 C>T* and *NFIB rs28379954 T>C* variants

Investigation of variants that may be in linkage disequilibrium with the *CYP1A-T* variant haplotype revealed two SNPs with moderate linkage, namely *rs2470893* ($D' = 1$; $R^2 = 0.66$) and *rs35107470* ($D' = 0.91$; $R^2 = 0.56$). The CYP1A1/1A2 promotor polymorphism *rs2470893* was shown to be a genetic determinant of 8% of variation in caffeine pharmacokinetics reflecting CYP1A2 activity,[29] higher aryl hydrocarbon receptor-xenobiotic response element binding, and CYP1A1 mRNA expression compared with homozygous wild type allele carriers.[30] Furthermore, *rs35107470* was shown the be associated with metabolism of caffeine, which is a CYP1A2 substrate.[21] In line with our recent study, we did not observe other genetic variants that are in linkage disequilibrium with the *NFIB-C* variant.[31]

DISCUSSION

The current study shows that the investigated *CYP1A* and *NFIB* variant alleles have significant and additive effects on dose-adjusted reduction of clozapine concentrations, and hence associated with increased dose requirements to reach target serum concentrations. The clinical relevance of these findings is likely highest among smokers, where clozapine metabolism is increased via enzyme induction. However, the quantitative effects of the genetics variants are also of significant importance in nonsmokers.

Currently, there is no reliable pharmacogenetic biomarker to predict clozapine serum levels prior to initiation of drug therapy. Tobacco smoking is the most important nongenetic factor of importance for individual dose requirements of clozapine and has been reported to reduce clozapine serum level by 30%–40%.[14] According to our results, patients who smoke and are carriers of both *CYP1A-T* and *NFIB-C* variant alleles exhibited a 66% reduction in clozapine serum levels, indicating an almost threefold increased dose requirement to reach the target therapeutic concentration range, compared with nonsmokers who do not carry either of the variant alleles. Thus, for patients who smoke, it would be of particular clinical importance to identify those being carriers of both *CYP1A/NFIB* variants, who represent about 2.5% of the clozapine-treated population. These patients would likely benefit from being prescribed higher clozapine doses than generally recommended both at treatment initiation and during dose titration to achieve the target concentration, and hence avoid delays in obtaining the optimal therapeutic effect. Therefore, pre-emptive genotyping of *NFIB* and *CYP1A* may be considered, alongside with information on smoking habits, as a clinical tool for precision dosing of clozapine during treatment initiation.

In current guidelines, TDM of clozapine is strongly recommended to prevent side effects and ascertain optimal systemic exposure.[6,7] A serum concentration of 1070 nmol/L (350 ng/ml) is considered as the lower boundary of the therapeutic range to obtain satisfactory clinical effect of clozapine, hence the dose should be titrated to exceed this serum level cutoff before augmentation strategies are considered.[5–8] This is supported by a current meta-analysis of clozapine serum levels and therapeutic response rates by Siskind et al.,[9] where clozapine serum levels below the recommended lower therapeutic threshold were significantly associated with lower therapeutic response and higher relapse rates during clozapine therapy. In our study population, patients who smoke carrying both variants exhibited an almost threefold higher frequency clozapine levels below the target concentration range compared with nonsmoker noncarrier patients, which underscores the clinical relevance of the present findings.

TABLE 2 Estimated means of dose-adjusted and absolute serum concentration of clozapine, and *N*-desmethylclozapine-to-clozapine ratio according to *CYP1A rs2472297 C>T* and *NFIB rs28379954 T>C* genotypes and smoking habits

Genotype	Smokers, $N = 335$				Non-smokers, $N = 191$			
	n, subject (sample)	Estimated mean (95% CI)	Fold change (95% CI)[a]	*p* Value[a]	*n*, subject (sample)	Estimated mean (95% CI)	Fold change (95% CI)[a]	*p* Value[a]
Dose-adjusted serum concentration (nmol/L/mg/day)								
CYP1A C/T - NFIB T/C	13 (185)	1.52 (1.16, 1.99)	0.52 (0.39, 0.68)	<0.0001	7 (124)	2.99 (2.06, 4.33)	0.65 (0.44, 0.95)	0.028*
CYP1A C/C - NFIB T/C	24 (232)	2.39 (1.95, 2.92)	0.81 (0.65, 1.01)	0.059	12 (126)	3.86 (2.92, 5.11)	0.84 (0.62, 1.13)	0.24
CYP1A C/T - NFIB T/T	119 (1688)	2.47 (2.26, 2.71)	0.84 (0.75, 0.94)	0.003	74 (1144)	3.93 (3.51, 4.41)	0.85 (0.73, 0.99)	0.042*
CYP1A C/C - NFIB T/T	179 (2688)	2.94 (2.73, 3.18)	–	–	98 (1723)	4.61 (4.17, 5.09)	–	–
All genotypes	335 (4793)	2.65 (2.50, 2.80)	0.63 (0.57, 0.69)[b]	<0.0001[b]	191 (3117)	4.24 (3.94, 4.55)	–	–
Absolute serum concentration (nmol/L)								
CYP1A C/T - NFIB T/C	13 (185)	564 (428, 744)	0.52 (0.39, 0.69)	<0.0001	7 (124)	917 (625, 1347)	0.66 (0.45, 0.98)	0.042*
CYP1A C/C - NFIB T/C	24 (232)	877 (712, 1080)	0.80 (0.64, 1.00)	0.055	12 (126)	1164 (871, 1554)	0.84 (0.62, 1.14)	0.26
CYP1A C/T - NFIB T/T	119 (1688)	962 (878, 1055)	0.88 (0.79, 0.99)	0.036*	74 (1144)	1165 (1035, 1312)	0.84 (0.72, 0.98)	0.031*
CYP1A C/C - NFIB T/T	179 (2688)	1091 (1009, 1180)	–	–	98 (1723)	1386 (1250, 1536)	–	–
All genotypes	335 (4793)	934 (882, 990)	0.67 (0.61, 0.73)[b]	<0.0001[b]	191 (3117)	1399 (1300, 1506)	–	–
***N*-desmethylclozapine-to-clozapine ratio**								
CYP1A C/T - NFIB T/C	12 (177)	0.78 (0.67, 0.92)	1.19 (1.01, 1.40)	0.037*	7 (104)	0.68 (0.56, 0.83)	1.14 (0.93, 1.39)	0.21
CYP1A C/C - NFIB T/C	24 (223)	0.63 (0.56, 0.71)	0.96 (0.85, 1.09)	0.52	12 (101)	0.59 (0.51, 0.68)	0.98 (0.83, 1.14)	0.76
CYP1A C/T - NFIB T/T	117 (1632)	0.69 (0.66, 0.73)	1.06 (0.99, 1.13)	0.09	73 (1106)	0.63 (0.59, 0.66)	1.04 (0.96, 1.12)	0.36
CYP1A C/C - NFIB T/T	178 (2616)	0.66 (0.63, 0.69)	–	–	94 (1645)	0.60 (0.57, 0.63)	–	–
All genotypes	331 (4648)	0.67 (0.65, 0.69)	1.10 (1.04, 1.15)[b]	0.0002[b]	189 (2956)	0.61 (0.59, 0.64)	–	–

Note: Estimated means were derived from linear mixed model analysis with age, gender, and withdrawal time as covariates. For estimation of absolute serum concentration of clozapine, total daily dose was also added as a covariate. All mixed model analysis were performed in log-transformed dependent variables; however, results are transformed into linear scale for representation. Conversion factor for clozapine serum levels from nmol/L to ng/ml: 1/3.06.

Abbreviation: CI, confidence interval.

[a]Unadjusted *p* values for estimates of variant allele carriers were compared with homozygous wild-type allele carriers in each smoking habit cohort.

[b]Estimates of smokers were compared with nonsmokers regardless of *CYP1A rs2472297* or *NFIB rs28379954* genotype.

*$p > 0.05$ after correction for multiple comparisons with Dunnett's test.

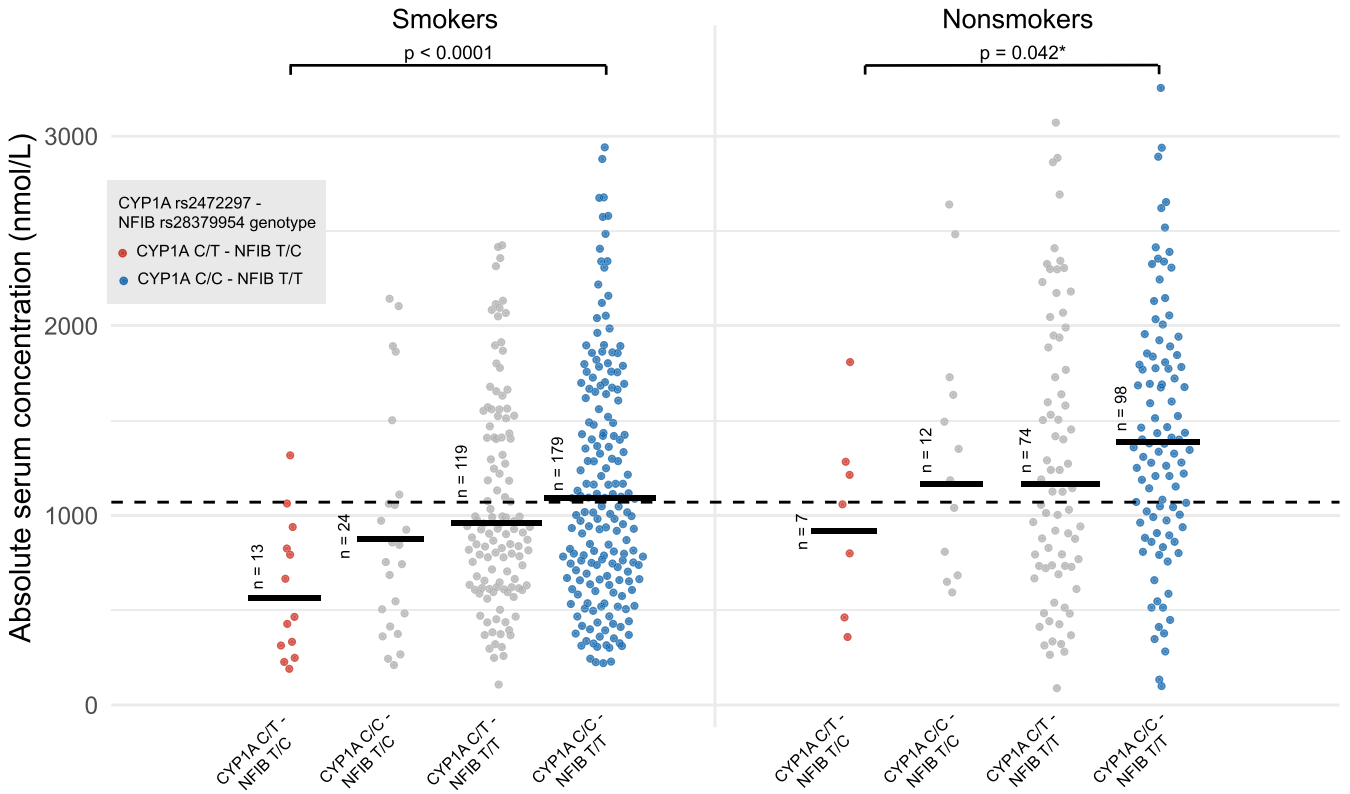

FIGURE 1 Influence of the *CYP1A-T* and *NFIB-C* variants on absolute clozapine serum concentration according to smoking habits. Each point represents the median clozapine serum concentration for each patient. Horizontal black bars represent estimated marginal means derived from linear mixed model analysis. The horizontal black dashed line represents the lower boundary of therapeutic reference range defined by AGNP group (1070 nmol/L [350 ng/ml]).[6] *$p > 0.05$ after multiple comparisons adjustments

The present study using targeted genotyping confirmed the significant effect of the *CYP1A-T* variant on clozapine concentration reported in the GWAS by Pardiñas et al.[18] Similar estimations of *CYP1A-T* on clozapine serum levels as in Pardiñas et al.'s GWAS and consistent additive variant effects observed in paired gene association analyses in patient subgroups in the present study are reassuring for validity of our results. Pardiñas et al.[18] used a proxy measure for smoking habits and did not observe any significant association between the *NFIB-C* variant and clozapine concentration. In our less-powered GWAS, we had exact data on individual smoking habits and found the *NFIB-C* variant to be significantly associated with clozapine serum levels.[23] This points to the importance of including well-controlled phenotypes in genetic studies for discovery of novel variants and underscore how important environmental factors are for understanding pharmacogenetic factors.

In the current study, we had access to measurements of *N*-desmethylclozapine. The key enzymes mediating clozapine *N*-demethylation are CYP1A2 and CYP3A4.[17] Just recently, we reported that NFIB is involved in the regulation of several ADME genes, including *CYP1A* but not *CYP3A4*.[31] Moreover, nuclear factor I (probably related to NFIB) has been shown to play a role in regulating *CYP1A* expression by mediating aryl hydrocarbon receptor induction cascade in response to oxidative stress.[32] This may cast light on why carriers of both *CYP1A-T* and *NFIB-C* variants had a moderate but significantly increased *N*-desmethylclozapine-to-clozapine metabolic ratio in smokers only. Our interaction analyses did not provide any evidence for potential epistasis between *NFIB* and *CYP1A* variants on clozapine serum concentration or metabolism. This may be due to a limited population of variant allele carriers for investigating interaction analyses and larger populations may be necessary. Nuclear factor I family transcription factors are involved in regulation of gene expression in different cellular systems.[33] Thus, the remaining unexplained clearance in variant allele carriers may be due to NFIB playing a role in the expression of genes mediating metabolism of clozapine via other metabolic pathways. Additional research is required to elucidate the exact mechanism of NFIB in the regulation of clozapine metabolism.

One of the limitations of the present study is the lack of information about clinical severity, comorbidity, treatment outcome, or side effects. Nonadherence to treatment is always an issue in naturalistic medication studies and may bias the assessments in intraindividual variability in clozapine serum levels between patient subgroups. However, the adherence rate of clozapine is shown to be high, probably due to the frequent hematological and

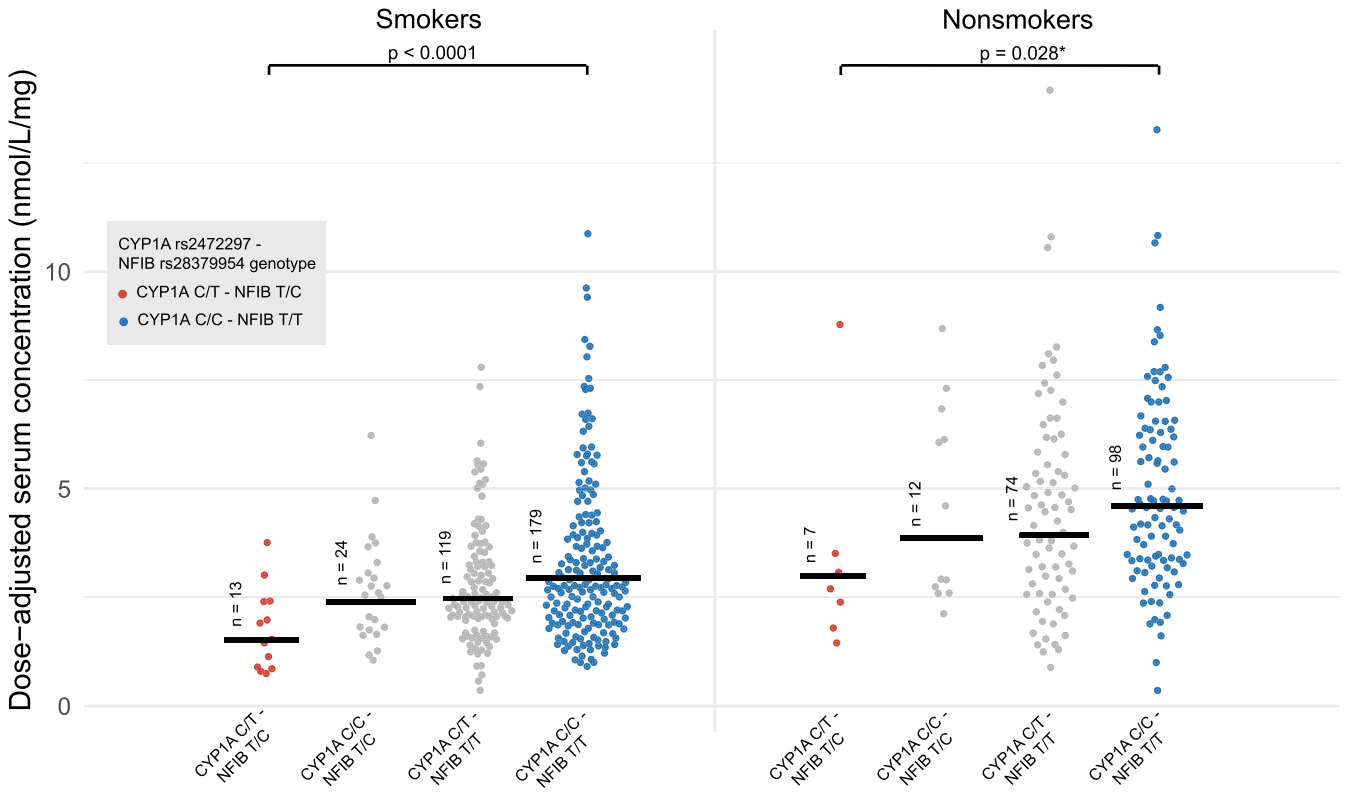

FIGURE 2 Influence of the *CYP1A-T* and *NFIB-C* variants on dose-adjusted clozapine serum concentration according to smoking habits. Each point represents the median dose-adjusted serum concentration of clozapine for each patient. The horizontal black bars represent estimated marginal means derived from linear mixed model analysis. *$p > 0.05$ after multiple comparisons adjustments

TABLE 3 Comparisons of patient proportions with subtherapeutic clozapine serum levels according to *CYP1A rs2472297 C>T* and *NFIB rs28379954 T>C* genotypes and smoking habits

		Smokers, $N = 335$			Nonsmokers, $N = 191$		
Genotype	**Overall, *n*; subject (%)**	***n*, subject (%)**	**Odds ratio (95% CI)**	***p* Value[a]**	***n*, subject (%)**	**Odds ratio (95% CI)**	***p* Value[a]**
Patients with subtherapeutic serum levels							
All genotypes	274 (52)	198 (59)	2.2 (1.5, 3.2)	<0.0001[b]	76 (40)	–	–
CYP1A C/T - NFIB T/C	16 (80)	12 (92)	10.8 (1.5, 468.2)	0.007	4 (57)	2.9 (0.5, 20.7)	0.22
CYP1A C/C - NFIB T/C	23 (64)	18 (75)	2.7 (1.0, 8.7)	0.049*	5 (42)	1.5 (0.4, 6.2)	0.52
CYP1A C/T - NFIB T/T	110 (57)	74 (62)	1.5 (0.9, 2.5)	0.12	36 (49)	2.0 (1.1, 4.0)	0.028*
CYP1A C/C - NFIB T/T	125 (45)	94 (53)	–	–	31 (32)	–	–

Note: Patients were classified as having subtherapeutic clozapine serum levels at median absolute clozapine serum concentration measurements were below than the lower therapeutic threshold (1070 nmol/L [350 ng/ml]). Proportions were compared with Fisher's exact test.

Abbreviation: CI, confidence interval.

[a]Estimates of variant allele carriers were compared with homozygous wild-type allele carriers in each smoking habit cohort.

[b]Estimates of smokers were compared with nonsmokers regardless of *CYP1A rs2472297* or *NFIB rs28379954* genotype.

*$p > 0.05$ after Bonferroni correction for multiple comparisons.

concentration monitoring.[34] Furthermore, we excluded measurements with nondetectable clozapine serum concentrations to avoid measurements probably reflecting complete nonadherence. Another potential limitation is that co-medication information is gathered from requisition forms filled by physicians, thus there may be missing co-medications potentially interacting with clozapine metabolism. However, the most relevant interacting co-medications are enzyme-inducing anti-epileptic drugs and fluvoxamine, which are usually listed on the requisition forms or subjected to TDM along with clozapine. Measurements registered with these co-medications were excluded from the study. We did not have relevant information on body mass index of patients or organ pathologies,

which may affect clozapine pharmacokinetics. However, the above-mentioned limitations may be outweighed by the large real-world populations that can effectively be included by using existing TDM data of genotyped patients, as for the current pharmacogenetic study. Furthermore, availability of multiple serum concentrations and information on key confounders, particularly smoking habits, are favorable for precise estimations of the effects of variant genotypes on clozapine serum levels.

In summary, carriers of the *CYP1A-T* and *NFIB-C* variant alleles had significant and additive effects on the dose-adjusted reduction in clozapine serum levels, which may be of special clinical relevance in smokers. Thus, patients with these genotypes who also are smokers (about 2.5% of the study population), are at high risk of subtherapeutic serum levels of clozapine and may require higher doses than generally recommended to reach the target serum concentration threshold and prevent clozapine undertreatment. Pre-emptive genotyping of *NFIB* and *CYP1A*, alongside with access to smoking status, would therefore constitute a clinical tool for precision dosing of clozapine during treatment initiation to ensure optimal serum levels and improve treatment outcomes for patients with TRS.

AUTHOR CONTRIBUTIONS

H.Ç.L., R.L.S., K.S.O., M.M.J., M.K.K., O.A.A., M.I.-S., and E.M. wrote the manuscript. E.M. and M.I.-S. designed the research. H.Ç.L. performed the research. H.Ç.L. and R.L.S. analyzed the data.

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CONFLICT OF INTEREST

Ole A. Andreassen has received a speaker's honorarium from Lundbeck and is a consultant for HealthLytix. Magnus Ingelman-Sundberg is a co-founder and co-owner of HepaPredict AB. All other authors declared no competing interests for this work.

DATA AVAILABILITY STATEMENT

All raw and processed data are available upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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