Original Paper

Contributions of cholinergic receptor muscarinic 1 and CYP1A2 gene variants on the effects of plasma ratio of clozapine/N-desmethylclozapine on working memory in schizophrenia

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Abstract

Background: Clozapine has heterogenous efficacy in enhancing working memory in schizophrenia. We have previously hypothesized that this is due to opposing effects of clozapine and its metabolite, N-desmethylclozapine, at the muscarinic M1 receptor and demonstrated that a lower clozapine/*N*-desmethylclozapine ratio is associated with better working memory than clozapine or *N*-desmethylclozapine levels alone.

Aims: In this study, we expanded the above hypothesis to explore whether genetic variation in the cholinergic receptor muscarinic 1 gene, encoding the M1 receptor, affects the relationship between clozapine/*N*-desmethylclozapine and working memory. Further, we explored whether CYP1A2 gene variants affect the ratio of clozapine/*N*-desmethylclozapine and by this, working memory performance.

Methods: We evaluated two functionally significant single nucleotide polymorphisms, rs1942499 and rs2075748, in cholinergic receptor muscarinic 1, with the haplotype T-A associated with lower transcriptional activity than the haplotype C-G. Further, we examined *CYP1A2* *1F, with *1F/*1F conferring high inducibility in the presence of smoking.

Results: In a sample of 30 patients with schizophrenia on clozapine monotherapy, clozapine/*N*-desmethylclozapine was correlated with working memory only in non-carriers of the haplotype T-A of the *cholinergic receptor muscarinic 1* gene. Interaction of *CYP1A2* genotype and smoking status significantly affected clozapine concentrations, but there were no significant effects of *CYP1A2* genotype and smoking status on the relationship between clozapine/*N*-desmethylclozapine on working memory.

Conclusions: Our finding that the relationship between clozapine/*N*-desmethylclozapine and working memory is specific to patients with potentially higher transcription of M1 receptor (i.e. non-carriers of the haplotype T-A of *cholinergic receptor muscarinic 1*) supports a cholinergic mechanism underlying this relationship.

Keywords

CHRM1, CYP1A2, clozapine, N-desmethylclozapine, schizophrenia, working memory

Introduction

Cognitive dysfunctions are strongly related to functional outcomes in schizophrenia (SCZ) (Bowie and Harvey, 2006; Green et al., 2004). Existing antipsychotic medications offer minimal benefit in treating these core features of the disorder (Harvey et al., 2004). Deficits in working memory, which is a cognitive process related to the short-term retention and manipulation of immediately relevant information, have been reliably observed in individuals with SCZ (Forbes et al., 2009; Lee and Park, 2005). As with other cognitive symptoms of SCZ, working memory deficits persist even after response to antipsychotic treatment. Clozapine (CLZ) is an antipsychotic that is employed as third-line or last resort treatment option for SCZ and has demonstrated undeniable superior efficacy for positive symptoms compared to other antipsychotics; however, its effects on cognitive symptoms, including working memory deficits, remain unclear. CLZ has demonstrated mixed effects on cognition in a number of studies, ranging from beneficial to neutral to deleterious (Nielsen et al., 2015; Rajji et al., 2015).

Previous studies have postulated that the unique effects of CLZ's principal metabolite, *N*-desmethylclozapine (NDMC), at the M1 muscarinic acetylcholine receptor (M1R) may be contributing

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to the mixed efficacy of CLZ on cognitive symptoms (Molins et al., 2017; Rajji et al., 2010, 2015; Weiner et al., 2004). *In vitro* and *in vivo* assays have shown NDMC behaving as a potent partial agonist at the M1R, whereas CLZ displayed potent M1R antagonist actions and was observed to antagonise NDMC-induced M1R responses (Li et al., 2005; Sur et al., 2003). Interestingly, the latter effects were observed in the medial prefrontal cortical and hippocampal sections, but not in sections from the nucleus accumbens; the former two sections are implicated in the mediation of cognitive processes, including executive function and memory (Li et al., 2005; Mendoza and Lindenmayer, 2009).

Given that activation of the M1R mediates procognitive effects (Bradley et al., 2010; Melancon et al., 2013), CLZ as an antagonist at the M1R is expected to worsen cognitive performance, whereas NDMC as an efficacious partial agonist of M1R is expected to improve cognition. In this vein, and given the role of the cholinergic system in working memory (Furey et al., 1997), we and others have previously shown the ratio of CLZ/NDMC is a better predictor of working memory performance than CLZ or NDMC alone in SCZ patients treated with CLZ monotherapy (Molins et al., 2017; Rajji et al., 2015). The smaller the ratio of CLZ/NDMC (i.e. more circulating NDMC in plasma compared to CLZ), the better the working memory performance (Rajji et al., 2015).

To further investigate whether the observed effects of CLZ/NDMC on working memory are mediated by M1R in patients with SCZ, we conducted the present secondary analysis using the same sample of CLZ treated patients that we previously published on (Rajji et al., 2015). In this study, we assessed the role of genetic variations in the cholinergic receptor muscarinic 1 *(CHRM1)* gene, which encodes M1R, in the relationship between CLZ/NDMC and working memory. Alterations to normal M1R expression in cortical and subcortical regions have been consistently reported in studies using post-mortem brain samples (Scarr and Dean, 2008), with one reporting significantly decreased *CHRM1* cDNA levels in cortical regions central to the pathophysiology of SCZ (Mancama et al., 2003). It is possible that genetic variations in *CHRM1* may be contributing to altered enzyme expression and function in patients with SCZ. This postulation is further corroborated by results from a functional study using luciferase-based reporter plasmids expressing two *CHRM1* haplotypes (C-G and T-A formed by single nucleotide polymorphisms (SNPs) rs2075748 and rs1942499) in the promoter transfected into human neuroblastoma IMR32 cells (Maeda et al., 2006). The reporter plasmid expressing the haplotype T-A in the promoter region demonstrated 37% lower transcriptional activity compared to the reporter plasmid carrying the haplotype C-G promoter (Maeda et al., 2006). Therefore, assuming that being a carrier of the haplotype T-A of *CHRM1* SNPs rs2075748 and rs1942499 is associated with lower expression of M1R than being a non-carrier, we explored the hypothesis that CLZ/NDMC interacts with M1R to affect working memory performance, therefore any variations in M1R expression will also affect working memory outcome. Specifically, only the T-A non-carrier patients will show an effect of CLZ/NDMC ratio on working memory, as the carriers would have less MR1 expression and, in turn, would be less susceptible to the impact of CLZ/NDMC ratio on cognition.

Additionally, because the concentrations of NDMC has been reported to vary from 20–150% of CLZ levels in plasma (Bondesson and Lindström, 1988), circulating levels of NDMC and CLZ in plasma available to bind to the M1R may also contribute to observed mixed efficacy of CLZ on cognition in individuals with SCZ. Genetic variations observed in pharmacokinetic gene, *CYP1A2*, which encodes the major hepatic cytochrome P450 1A2 (CYP1A2) enzyme involved in the oxidative biotransformation of CLZ to NDMC, may be contributing to large variances observed in plasma concentrations of CLZ and NDMC and their ratio. Furthermore, polycyclic aromatic hydrocarbons in tobacco smoke and caffeine are common inducers of CYP1A2, with *CYP1A2**1F gene variant (rs762551) conferring increased enzyme inducibility (Gunes and Dahl, 2008). The *CYP1A2**1F gene variant, leading to a C to A transition located in intron 1 at position 734 downstream from the transcriptional initiation site, has been reported to affect the pharmacokinetics of CLZ. As such, smokers with *1F/*1F genotype have a 60–70% larger increase in CYP1A2 activity than individuals with *1/*1 and *1/*1F genotypes (Sachse et al., 1999). Because individuals with SCZ have a high prevalence of smoking compared to the general population with rates ranging from 60–80% (Yee et al., 2015), the presence of *CYP1A2**1F allele and smoking behaviour may impact the plasma CLZ/NDMC ratio and ultimately cognitive outcome in response to CLZ pharmacotherapy. Thus, we explored whether there will be an interaction between *CYP1A2* genotype and smoking behaviour on the concentrations of CLZ, NDMC and CLZ/NDMC ratio in plasma, and we further explored the influence of this interaction on the relationship between CLZ/NDMC ratio and working memory.

Material and methods

Participants

A detailed description of the study sample and methods have been previously described in Rajji et al. (2015). In total, 30 participants meeting the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision criteria for SCZ or schizoaffective disorder (age ranging from 21–70), recruited from the Centre for Addictions and Mental Health (CAMH) in the Greater Toronto Area, were stable on CLZ monotherapy taken once daily. For inclusion into the study, participants were required to have no history of hospitalisation within the past 3 months and needed to be on stable CLZ dosage for at least 4 weeks. Diagnosis was confirmed using Mini International Neuropsychiatric Interview and clinical symptoms were assessed with the Positive and Negative Syndrome Scale. The study protocol was approved by the CAMH Research Ethics Board and written informed consent was obtained from all participants.

Working memory assessments

Working memory was assessed using the Measurement and Treatment Research to Improve Cognition in Schizophrenia Consensus Cognitive Battery (MCCB) on the day blood was collected from participants. MCCB includes 10 cognitive tests measuring seven cognitive domains of which two tests measure working memory domain: the letter-number span test (LNST) and the spatial span subtest of the Wechsler Memory Scale-III (SSP). LNST is a measure of verbal learning that involves the

participant repeating correctly reordered letter-number strings of increasing length back to the test administrator (Holmén et al., 2010). SSP is a measure of non-verbal memory consisting of the total number of correct responses demonstrated by the participant tapping a board of irregularly spaced blocks in the same or reverse sequence as the test administrator (Holmén et al., 2010). A composite measure of working memory using LNST and SSP was generated as per MCCB guidelines and was used as the primary cognitive outcome in this study as it was in our previous publication (Rajji et al., 2015). The remaining MCCB tests were also administered to assess functioning on the other cognitive domains: speed of processing, attention/vigilance, verbal learning, visual learning, reasoning and problem solving, and social cognition. *T*-scores for all cognitive domains and their subtests were corrected for age and sex.

Plasma concentrations

High-performance liquid chromatography with isocratic elution and ultraviolet detection at 245 nm was used to measure CLZ and NDMC levels in heparinized plasma with a limit of quantitation at 100 nmol/L, as described in Rajji et al. (2015). Smoking status (smoker vs. non-smoker) was determined based on the presence of cotinine levels in plasma.

Genotyping

Genomic DNA was extracted from a buffy coat. *CHRM1* rs2075748 and *CYP1A2**1F polymorphism (rs762551) were genotyped as per the manufacturer's directions for commercially available TaqMan® SNP genotyping assays. The *CHRM1* rs1942499 variant was genotyped using standard BigDye® Sanger sequencing procedures. The primers used to amplify and sequence the region surrounding rs1942499 were developed in-house.

Biological sex was determined using a custom TaqMan® SNP genotyping assay specific for a region in the amelogenin gene that differs between the X and Y chromosomes. Further details on the genotyping methods can be obtained from the corresponding author.

Statistical analysis

Haploview software program version 4.2 (http://www.broad.mit.edu/personal/jcbarret/haploview/) was used to determine the Hardy-Weinberg equilibrium, linkage disequilibrium and allele, genotype and haplotype frequencies of the *CHRM1* and *CYP1A2* SNPs of interest as appropriate.

Statistical analysis was performed using R software version 4.0.0. Normal distribution of variables was determined using the Shapiro-Wilk test. We analysed categorical variables using Fisher's exact test and an χ^2-test as appropriate. Differences of quantitative variables between genotype groups were evaluated using Welch's *t*-tests and nonparametric tests (Mann-Whitney *U*-test or Kruskal-Wallis test) as appropriate.

For the primary analysis exploring whether genetic variation in the *CHRM1* gene affects the relationship between CLZ/NDMC and working memory, Spearman's correlations were conducted within each *CHRM1* haplotype group separately, between CLZ/NDMC ratio and working memory composite *T*-score, as well as between CLZ/NDMC ratio and the two working memory subtests individually (LNST and SSP).

Additionally, post-hoc Spearman's correlations were performed to determine the effect of the *CHRM1* haplotype group (T-A carriers and non-carriers) on the relationship between plasma CLZ or NDMC concentrations on working memory performance for comparisons with the findings from the primary analysis using CLZ/NDMC ratio.

For the exploratory pharmacokinetics analyses, a Kruskal-Wallis test was used to investigate differences in plasma CLZ and NDMC concentrations and in CLZ/NDMC ratio between the four groups: *1/*1 and *1/*1F non-smokers and smokers and *1F/*1F non-smokers and smokers. Post hoc two-sample Mann-Whitney *U* tests were conducted to decompose any significant effects. Further, the impact of the *CYP1A2* gene and smoking status on the relationship between concentrations variables and working memory was explored using Spearman's correlations.

For all analyses, two-tailed p values <0.05 were considered statistically significant.

Results

Sample characteristics

Demographic, clinical and pharmacological characteristics of the sample, as well as working memory performance and performance on the other cognitive domains, are listed in Table 1. Two participants did not have sufficient DNA samples for the genotyping of *CHRM1* and *CYP1A2* SNPs of interest, and one participant did not have a remaining DNA sample to genotype *CYP1A2* following *CHRM1* genotyping. Therefore, statistical analyses involving *CHRM1* and *CYP1A2* included 28 and 27 participants, respectively.

Within the total sample ($n=30$), patients had a mean age of 38.6 (SD = 15.3) years and were predominantly male ($n=18$, 60%). The ethnic composition of the sample was 78% white and 22% non-white, although one participant's ethnicity is unknown. The CLZ daily dose varied between 150 and 550 mg (mean ± SD: 352.7 ± 105.1 mg). For *CHRM1* ($n=28$), the dose for CLZ was significantly lower in haplotype T-A non-carriers than those who are carriers (314.0 ± 94.5 versus 422.5 ± 101.7; $p=0.01$). Haplotype T-A non-carriers also demonstrated significantly better performance for the MCCB reasoning and problem-solving cognitive domain compared to carriers (37.4 ± 8.8 versus 30.2 ± 3.7; $p=0.02$). No significant differences were observed for all other variables between *CHRM1* haplotype T-A non-carriers and carriers. There were no significant differences in any of the above-mentioned characteristics between *CYP1A2* genotype groups (Supplementary Table 1).

Haplotype, genotype and allele frequencies for CHRM1 and CYP1A2

CHRM1 SNPs rs2075748 and rs1942499 were in a Hardy-Weinberg equilibrium. The two SNPs have a *D*' of 1.00 and r^2 of 0.107. Haplotypes C-A, C-G and T-A formed by SNPs rs2075748 and rs1942499 have frequencies of 0.500, 0.304 and 0.196, respectively. Allele and genotype counts and minor allele frequencies for *CHRM1* and *CYP1A2* are listed in Supplementary Table 2.

Table 1. Basic sample demographics and clinical information compared between non-carriers and carriers of haplotype T-A formed by *CHRM1* SNPs rs2075748 and rs1942499.

Variable	Total (n=30)			*CHRM1* haplotype[a]						Statistics (df)	p	Effect size	95% CI
				Non-carriers (n=18)			Carriers (n = 10)						
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range				
Sex (female: male)	12:18	-	-	10:8	-	-	2:8	-	-	$\chi2(1)=2.03$	0.15	0.07	(0.82, 30.46)
Diagnosis (schizophrenia: schizoaffective)	25:5	-	-	15:3	-	-	8:2	-	-	$\chi2(1)=4.93$	1.00	0.83	(0.17, 9.09)
Age in years	38.57	15.30	21–70	39.94	15.45	21–70	33.50	14.49	22–60	$W(27)=113$	0.28	0.43	(–4.00, 20.00)
Ethnicity (white: non-white)[b]	21:6	-	-	13:4	-	-	8:2	-	-	$\chi2(1)= 6.65\text{x}10^{-31}$	1.00	1.23	(0.18, 8.33)
Education in years	12.24	2.6	6–19	12.06	2.71	6–17	12.50	2.76	9–19	$t(18)=-0.41$	0.69	–0.16	(–2.71, 1.82)
Clozapine dose (mg/day)	352.68	105.11	150–550	314.04	94.47	150–525	422.5	101.69	250–550	$t(18)=-2.77$	**0.01**	–1.12	(–190.78, –26.14)
Clozapine concentration (nmol/L)	2317.79	1432.21	498–5864	2004.82	1326.72	498–5680	2619.8	1492.89	1134–5864	$W(27)=63$	0.21	–0.44	(–1637, 467)
NDMC concentration (nmol/L)	1608	1026.58	221–4765	1585.83	1218.31	221–4765	1647.9	767.71	627–2822	$W(27)=76$	0.52	–0.06	(–931, 655)
Clozapine/NDMC ratio	1.64	0.7	0.5–3.07	1.56	0.71	0.5–2.86	1.64	0.64	0.9–2.69	$t(20)=-0.28$	0.78	–0.11	(–0.62, 0.47)
Cotinine concentration (nmol/L)	75.71	147.73	0–523	54.78	100.12	0–305	113.4	209.96	0–523	$W(27)=83$	0.72	–0.40	(-0.53, $4.69\text{x}10^{-5}$)
Hydroxycotinine concentration (nmol/L)	31.25	65.31	0–225	26.78	60.13	0–225	39.30	76.52	0–217	$W(27)=87$	0.89	–0.19	(–11.00, 7.00)
PANSS													
Total	53.68	9.82	35–73	55.26	10.53	35–73	51.70	9.13	40–70	$t(21)=0.93$	0.36	0.35	(–4.36, 11.48)
Positive score	13.77	4.54	7–28	14.22	5.28	8–28	13.10	3.45	7–18	$t(25)=0.68$	0.50	0.24	(–2.28, 4.53)
Negative score	13.79	5	7–28	13.61	4.65	7–23	14.30	6.24	7–28	$t(15)= -0.31$	0.76	–0.13	(–5.51, 4.13)
General score	26.32	5.57	17–41	27.68	6.05	17–41	24.30	4.52	18–32	$t(24)=1.68$	0.11	0.61	(–0.79, 7.56)
MCCB *T*-scores on cognitive domains													
Working memory	35.97	9.15	21–50	36.56	9.14	23–50	35.60	8.88	25–50	$t(19)=0.27$	0.79	0.11	(–6.45, 8.36)
Speed of processing	34.69	12.73	7–67	36.15	15.02	7–67	32.00	9.06	13–46	$t(26)=0.91$	0.37	0.31	(–5.21, 13.51)
Attention/vigilance	34.73	12.14	16–58	36.83	12.07	16–58	32.70	12.45	17–51	$t(18)=0.85$	0.41	0.34	(–6.07, 14.33)
Verbal learning	35.47	9.12	23–62	35.56	8.81	25–62	35.10	10.25	23–59	$W(27)=100$	0.65	0.05	(–6.00, 7.00)
Visual Learning	30.63	12.64	4–61	30.06	11.13	4–49	29.50	12.97	12–50	$t(16)=0.11$	0.91	0.05	(–9.75, 10.86)
Reasoning and problem solving	35.10	7.89	22–55	37.44	8.79	22–55	30.20	3.74	24–37	$W(27)=140.5$	**0.02**	0.97	(2.00,12.00)
Social cognition	38.73	13.05	17–63	39.00	13.00	18–59	41.60	12.35	24–63	$t(20)=-0.52$	0.61	–0.20	(–12.97, 7.77)
Overall	25.66	11.79	8–57	27.04	13.39	8–57	23.8	9.48	10–41	$t(24)=0.74$	0.46	0.27	(–5.74, 12.22)

Comparisons between haplotype T-A non-carriers and carriers were conducted using Chi-square tests (sex, diagnosis), Mann–Whitney U tests (age, concentrations of clozapine, NDMC, cotinine and hydroxycotinine and verbal learning) or Welch's t-tests for normally distributed variables. Bold text indicates significant comparisons ($p < 0.05$). Effect sizes were calculated using Cohen's d for t-tests and Mann–Whitney U tests and OR for Chi-squared tests.

[a]Two participants did not have sufficient DNA sample for the genotyping of *CHRM1*.

[b]Ethnicity of one participant is unknown.

CHRM1: muscarinic acetylcholine receptor M1; MCCB: MATRICS consensus cognitive battery; NDMC: *N*-desmethylclozapine; PANSS: Positive and Negative Syndrome Scale; SD: standard deviation; CI: confidence interval; OR: odds ratio.

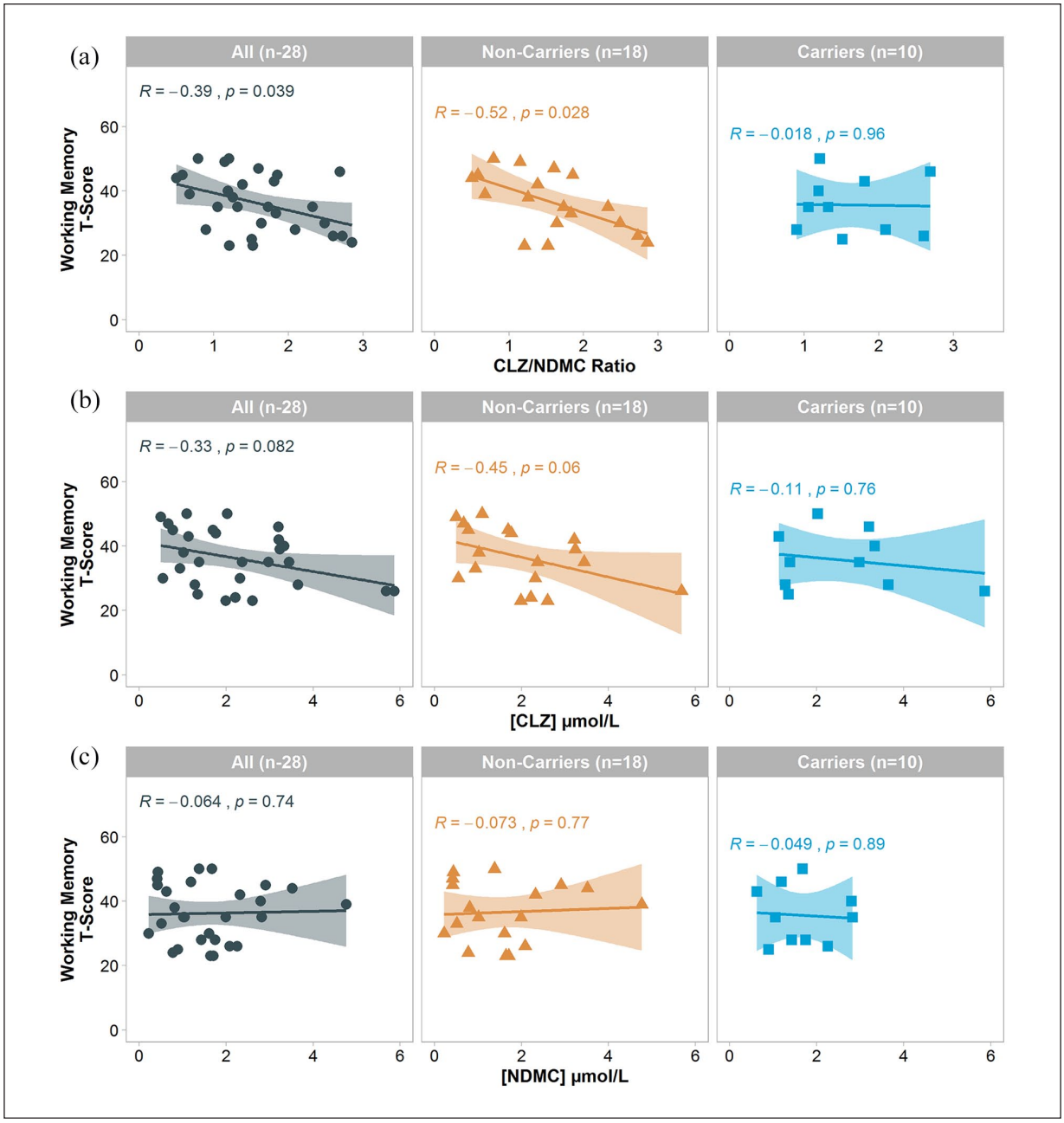

Figure 1. Correlations of CLZ/NDMC ratio, plasma CLZ and NDMC concentrations with composite working memory performance in haplotype T-A non-carriers and carriers formed by *CHRM1* single nucleotide polymorphisms (SNPs) rs2075748 and rs1942499. (a) A significant negative correlation was observed between CLZ/NDMC ratio and composite working memory *T*-scores in the whole sample and haplotype T-A non-carriers, with individuals with lower CLZ/NDMC ratio demonstrating better overall working memory performance. The correlation in T-A non-carriers was stronger than the correlation observed in the whole sample (p = 0.028 versus p = 0.039). (b) and (c) A significant correlation was not observed between CLZ and NDMC concentrations and working memory in any of the *CHRM1* haplotype groups.
CLZ: clozapine; *CHRM1*: muscarinic acetylcholine receptor M1; NDMC: N-desmethylclozapine.

CHRM1 effects on the correlation between CLZ and NDMC plasma concentrations and CLZ/NDMC ratio with working memory performance

Spearman correlations revealed that only the *CHRM1* haplotype T-A non-carriers showed a significant correlation between CLZ/NDMC ratio and composite working memory (r_s = -0.52, p = 0.028, 95% confidence interval (CI) = -0.79, -0.07) (Figure 1). This correlation in T-A non-carriers was stronger (n = 18, r_s = -0.52, p = 0.028, 95% CI = -0.79, -0.07) than the correlation observed in the whole sample (n = 28, r_s = -0.39, p = 0.039, 95% CI = -0.67, -0.02). Similarly, there was a significant correlation between CLZ/NDMC ratio and SSP (r_s = -0.57, p = 0.013, 95% CI -0.82, -0.15) only in the T-A non-carriers, which was not observed between CLZ/NDMC ratio and LNST (Supplementary Figures 1 and 2).

Further, no significant correlations between CLZ and NDMC concentrations with working memory measures were observed for either *CHRM1* haplotype groups. These results are consistent with our previous findings (Rajji et al., 2015).

CYP1A2 effects on concentration measures and working memory performance

CLZ and NDMC concentrations and CLZ/NDMC ratio in plasma did not significantly differ between *CYP1A2* *1/*1 and *1/*1F versus *1F/*1F genotype groups and between smokers versus non-smokers (Supplementary Tables 1 and 3).

Kruskal-Wallis tests revealed significant differences in CLZ concentrations (Chi square = 8.09, $p = 0.044$, $df = 3$) between the four groups formed by *CYP1A2* genotype and smoking status: (a) *1/*1 and *1/*1F smokers, (b) *1/*1 and *1/*1F non-smokers, (c) *1F/*1F smokers and (d) *1F/*1F non-smokers. Post hoc two-sample comparisons revealed smokers with *1F/*1F genotype have the lowest CLZ concentrations in plasma, which was significantly lower than *1F/*1F non-smokers ($W(8) = 20.0$, $Z = 2.45$, $p = 0.016$, 95% CI = 1214, 5009) (Figure 2(a)). In contrast, non-smokers with *1F/*1F genotype had the highest CLZ and NDMC concentrations in plasma compared to all other groups, with these levels being significantly higher when compared to *1/*1 and *1/*1F non-smokers ($W(16) = 4.0$, $Z = -2.66$, $p = 0.005$, 95% CI = -3077, -836). These effects were not due to CLZ dosage because the dose was not significantly different between all four *CYP1A2* genotype and smoking status groups (Supplementary Table 3). No significant differences in NDMC concentrations and CLZ/NDMC ratio were observed between the four groups (Figure 2(b)-(c)).

Further, Spearman correlations revealed no significant correlations between plasma levels of CLZ, NDMC and CLZ/NDMC ratio and composite working memory within any of the four groups.

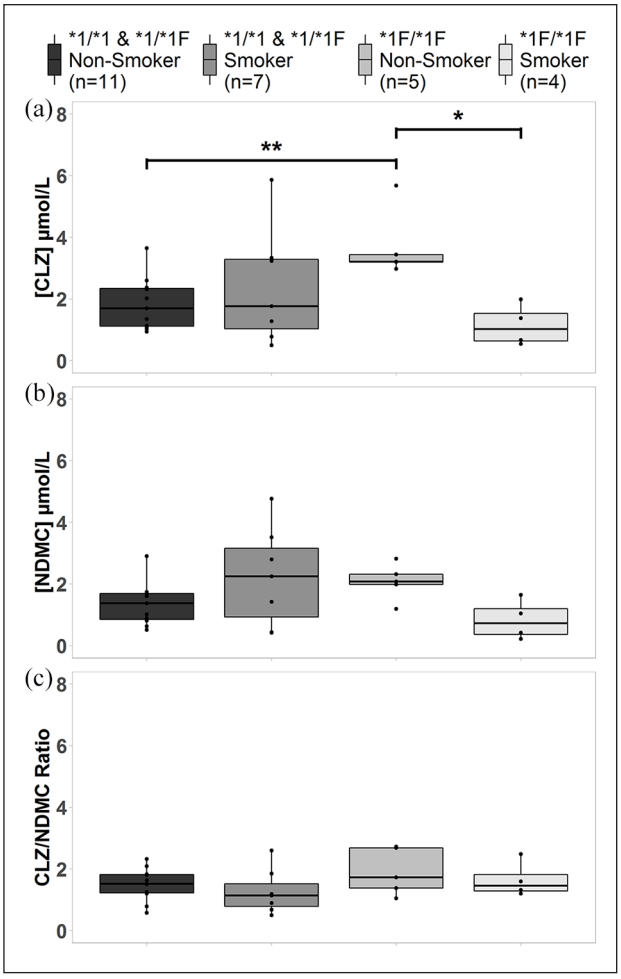

Figure 2. Association between *CYP1A2* genotype and CLZ and NDMC plasma concentrations and CLZ/NDMC ratio by smoking status. (a) Kruskal-Wallis tests revealed significant differences in plasma CLZ levels between groups (Chi square = 8.09, $p = 0.044$, $df = 3$). Simple main effects analyses showed that *1F/*1F non-smokers demonstrated a significantly higher CLZ plasma concentration compared *1F/*1F smokers ($p = 0.016$) and *1/*1 and *1/*1F non-smokers ($p = 0.006$). There were no significant differences in (b) NDMC plasma concentrations and (c) CLZ/NDMC ratio between the four groups. * Mann-Whitney *U* test $p<0.05$. **Mann-Whitney *U* test $p<0.01$. CLZ: clozapine; *CHRM1*: muscarinic acetylcholine receptor M1; NDMC: N-desmethylclozapine.

Discussion

This study was a secondary analysis based on a previously published study that showed a negative association between CLZ/NDMC ratio and working memory performance in patients with SCZ, but not with CLZ or NDMC alone (Rajji et al., 2015). The previous study was based on the premise that CLZ and NDMC impact cognition through their action on the M1R, but did not provide any evidence to support this premise. The current study demonstrates a similar negative correlation between CLZ/NDMC and working memory as observed in the previous study, but only in individuals lacking the haplotype T-A formed by *CHRM1* SNPs rs2075748 and rs1942499. Our results also show the two subgroups, T-A carriers and non-carriers, are indeed distinct because the correlation between CLZ/NDMC and working memory performance within the T-A non-carriers is stronger than the correlation we observed in the whole sample.

These findings were predicted a priori given that previous in vitro functional analysis has shown the plasmid expressing the haplotype T-A in the regulatory region was associated with a 37% decreased transcriptional activity compared with the plasmid expressing the haplotype G-C in IMR32 cells (Maeda et al., 2006). Thus, we posited that individuals expressing haplotype T-A would have decreased transcription of the *CHRM1* gene, leading to lower expression of M1R than individuals who do not carry this haplotype. For haplotype T-A carriers, as a result of having lower transcription and, consequently, expression of M1R, binding sites for CLZ and NDMC on the M1R are saturated before a detectable effect on working memory performance can be achieved. In turn, individuals who are non-carriers of this haplotype have normal transcriptional activity of the *CHRM1* gene and expression levels of M1R and as a result, they are more sensitive to CLZ/NDMC ratio leading to a more detectable effect on working memory performance. Taken together, the present findings provide support for our previous postulation that the mechanism through which CLZ and NDMC affected cognition in SCZ is via the muscarinic system.

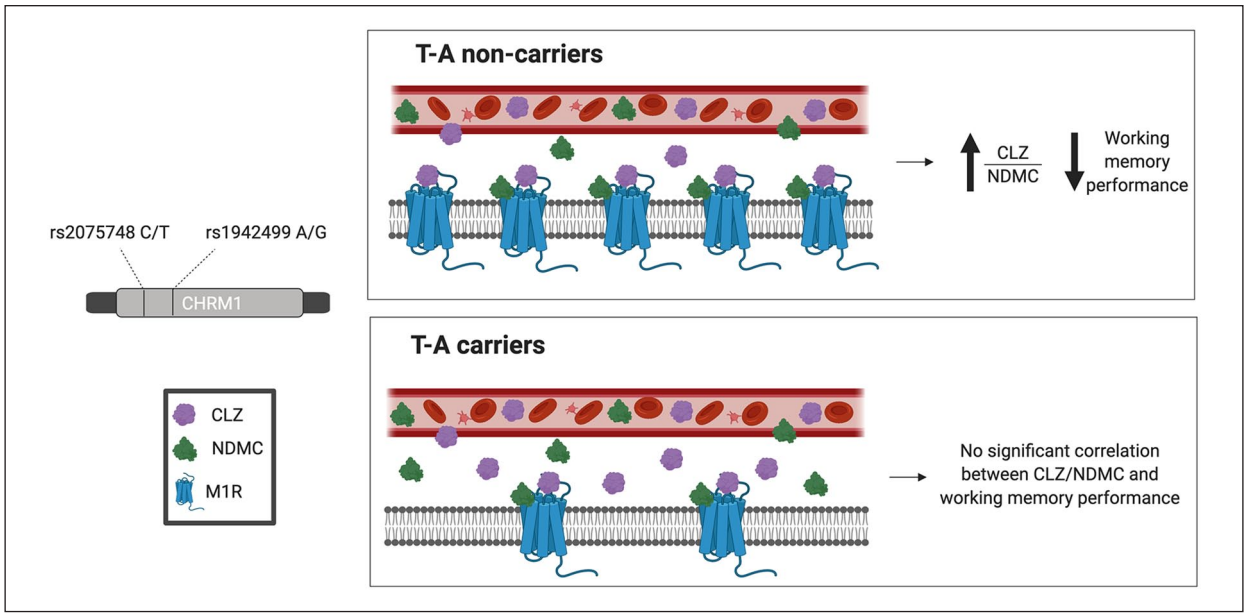

Figure 3. Hypothesised model for the effect of *CHRM1* rs2075748-T and rs1942499-A haplotype on the association between CLZ/NDMC ratio and working memory. Non-carriers of the *CHRM1* rs2075748-T and rs1942499-A haplotype have normal levels of transcription of the *CHRM1* gene and expression of the M1R, which leads to greater sensitivity to variations in CLZ/NDMC ratio, which consequently affects working memory processes. Carriers of *CHRM1* rs2075748-T and rs1942499-A haplotype have lower transcription of the *CHRM1* gene and expression of M1R, leading to faster saturation of CLZ and NDMC binding sites on the M1R prior to reaching clinically detectable effects of CLZ/NDMC ratio on working memory processes. *CHRM1*: muscarinic acetylcholine receptor M1; CLZ: clozapine; M1R: M1 muscarinic acetylcholine receptor; NDMC: N-desmethylclozapine. Created with BioRender.com.

Our hypothesized model for the influence of haplotype T-A formed by *CHRM1* on the relationship between CLZ/NDMC and working memory is shown in Figure 3. Based on pharmacological and site-directed mutagenesis studies on the pharmacodynamics of CLZ and NDMC, both CLZ and NDMC have been shown to activate M1Rs by interacting with sites that do not fully overlap with the M1R orthosteric site as does acetylcholine (Spalding et al., 2006; Sur et al., 2003). However, whether CLZ and NDMC bind to the same or overlapping sites on the M1R remains to be investigated. Therefore, the binding of CLZ and NDMC to M1R is depicted as not overlapping with each other or the orthosteric site in our hypothesised model.

Additionally, in this study we aimed to explore the contributions of genetic variations in pharmacokinetic gene, *CYP1A2*, to the effects of CLZ/NDMC ratio on working memory. Previous studies have reported an association between *CYP1A2 *1F/*1F* genotype with low CLZ plasma levels and non-response to CLZ (Balibey et al., 2011; Bender and Eap, 1998; Eap et al., 2004; Ozdemir et al., 2001). Furthermore, tobacco smoke constituents can induce CYP1A2 through the binding of polycyclic aromatic hydrocarbons to the aryl hydrocarbon receptor (AhR) leading to the transcriptional activation of the *CYP1A2* gene (Hukkanen et al., 2011). Our results corroborate these reports demonstrating an effect of *CYP1A2* genotype and smoking status on CLZ levels, with **1F/*1F* smokers showing lower CLZ levels than non-smokers of the same genotype. It is important to note there are environmental pollutants other than tobacco smoke, including polychlorinated biphenyls, which have affinity for AhRs and can contribute to regulating *CYP1A2* gene activity and as a result, may also affect CLZ metabolism (Hufgard et al., 2019).

CYP1A2 *1F/*1F non-smokers had significantly higher CLZ plasma concentrations than *1/*1 and *1/*1F non-smokers, although it was previously reported that there was no significant differences in CYP1A2 activity between non-smokers with *1/*1, *1/*1F and *1F/*1F genotypes (Sachse et al., 1999). The difference between our results and findings by Sachse et al. (1999) may be because the latter study measured CYP1A2 activity using urinary caffeine ratios, which the authors reported may not be a sensitive index (Sachse et al., 1999). One possibility is that individuals with *1F/*1F in the absence of an inducer are slightly slower metabolisers than those with *1/*1 and *1/*1F genotypes; however, additional investigation is required to resolve this discrepancy in findings.

One possible factor contributing to the differences in CLZ and NDMC plasma concentrations may be the differences in their stability. However, previous studies have shown that room temperature and frozen samples of both CLZ and NDMC demonstrate significant stability in plasma with little to no degradation occurring following 6 h bench-top storage and retention of stability following at least three freeze-thaw cycles without measurable decomposition higher than 10% (Guitton et al., 1997). Therefore, we can conclude that our results are not confounded by differences in the stability of CLZ or NDMC, which may have contributed to the differences in plasma concentrations of CLZ and NDMC.

Another factor to consider is sex differences in cognitive function in patients with SCZ, which are typically observed with male patients showing significant memory dysfunctions compared to female patients (Han et al., 2012). In our analysis, we have corrected working memory *T*-scores for age and sex. It is important for future studies to examine the effect of sex on the association between *CHMR1* and the relationship between CLZ/NDMC and working memory using a larger sample size.

The present findings have implications for the relevance of *CHRM1* and *CYP1A2* genotyping in patients on CLZ pharmacotherapy for the management of cognitive symptoms of SCZ. It is crucial that individuals with SCZ on CLZ or any other antipsychotic are offered clinical treatment that is proactive and individualized to improve long-term outcomes. Due to the high frequency of smokers among patients with SCZ and the high frequency of the *1F polymorphism, *CYP1A2* genotyping could potentially be beneficial for treating patients on CLZ who are smokers to ensure they receive clinically efficacious doses of the medication. Furthermore, because long-term functional outcome for individuals with SCZ is tied closely to improvements in cognitive symptoms (Bowie and Harvey, 2006; Green et al., 2004), pre-emptive *CHRM1* genotyping may aid physicians and patients in making decisions well in advance to help mitigate potential deficits in working memory associated with CLZ pharmacotherapy.

Limitations

Although the results of this study are promising, there are some limitations that should be considered. The primary limitation of this study is the small sample size, which could have contributed to a reduced ability to detect smaller genetic effects, thus increasing the risk of a false-negative result (i.e. type II error). Further, the small sample size after stratification by smoking status precludes making any definitive conclusions on the effects of *CYP1A2* on the relationship between CLZ/NDMC and working memory performance. Another limitation to consider is the heterogenous genetic ancestry of the study sample, which can be a cause of both false-positive and false-negative findings and can obscure true association signals. Additionally, ancestry is self-reported in this study, which is often unreliable in genetic association studies and limits assessments of variation within ancestral groups (Barnholtz-Sloan et al., 2008). Accordingly, these findings require further investigations in a larger and more ancestrally homogenous sample to validate and further explain the effect of *CHRM1* on the relationship between CLZ/NDMC and working memory that is observed in this study.

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