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Pharmacogenetic Analysis of Functional Glutamate System Gene Variants and Clinical Response to Clozapine

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Key Words
Glycine transporter 1 *(SLC6A9)* · Glutamate · Clozapine · Pharmacogenetics · Schizophrenia

Abstract
Altered glutamate neurotransmission is implicated in the etiology of schizophrenia (SCZ) and the pharmacogenetics of response to clozapine (CLZ), which is the drug of choice for treatment-resistant SCZ. Response to antipsychotic therapy is highly variable, although twin studies suggest a genetic component. We investigated the association of 10 glutamate system gene variants with CLZ response using standard genotyping procedures. *GRM2* (rs4067 and rs2518461), *SLC1A2* (rs4354668, rs4534557, and rs2901534), *SLC6A9* (rs12037805, rs1978195, and rs16831558), *GRIA1* (rs2195450), and *GAD1* (rs3749034) were typed in 163 European SCZ/schizoaffective disorder patients deemed resistant or intolerant to previous pharmacotherapy. Response was assessed following 6 months of CLZ monotherapy using change in Brief Psychiatric Rating Scale (BPRS) scores. Categorical and continuous response variables were analyzed using χ^2 tests and analysis of covariance, respectively. We report no significant associations following correction for multiple testing. Prior to correction, nominally significant associations were observed for *SLC6A9*, *SLC1A2*, *GRM2*, and *GRIA1*. Most notably, CC homozygotes of rs16831558 located in the glycine transporter 1 gene *(SLC6A9)* exhibited an allele dose-dependent improvement in positive symptoms compared to T allele carriers ($p_{uncorrected} = 0.008$, $p_{corrected} = 0.08$). To clarify the role of *SLC6A9* in clinical response to antipsychotic medication, and CLZ in particular, this finding warrants further investigation in larger well-characterized samples.

Introduction

The glutamate system has emerged as an area of strong interest in both schizophrenia (SCZ) risk and response to antipsychotic (AP) medications [1]. The glutamate hypothesis of SCZ first emerged from observations that ad-

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ministration of N-methyl-D-aspartate glutamate receptor (NMDAR) antagonists such as phencyclidine and ketamine elicit behaviors that mimic the symptoms of SCZ in healthy individuals and dramatically worsen symptoms in SCZ patients [2–5]. Glutamate levels also appear to be altered in SCZ brains, and dysregulation of glutamate receptors has been postulated to play an integral role in the hyperdopaminergic states often thought to be the primary cause of psychosis [6].

Clozapine (CLZ) is an atypical AP drug that is particularly effective for treating patients with treatment-resistant SCZ (TRS) [7]. Approximately 30% of patients fail two or more trials with AP drugs, and are subsequently diagnosed with TRS. For these individuals, CLZ is the one drug that has been approved for treatment of this particular subpopulation of patients [8–10]. Response to CLZ is complex and thought to depend, at least in part, on genetic factors, as indicated by twin and family studies [11–14]. Like other APs, high interindividual variability is also observed with CLZ treatment [15–17], with up to 50% of eligible patients failing to respond [7, 18]. Studies in pharmacogenetics aim to identify gene variants with the potential to predict dosing, response, and side effects prior to starting drug treatment [19]. The implications of a genetic test incorporating such variants would have far-reaching clinical applications; given the fact that early and effective treatment has been associated with favorable outcome [20, 21]. To date, the majority of pharmacogenetic studies investigating CLZ response have focused on gene variants in dopamine and serotonin neurotransmitter pathways [reviewed in 22].

As noted, there is mounting evidence to suggest that glutamatergic neurotransmission may also play a role in mediating response to CLZ [23, reviewed in 24]. The main observation supporting this hypothesis is that if CLZ is coadministered with NMDAR antagonists, their psychotomimetic effects are blunted [25]. Glutamate concentrations and excitatory glutamate neurotransmission are also increased by CLZ administration, as shown by microdialysis studies in rodents [26, 27], excitatory postsynaptic potentials in neuronal cell cultures [28–32], and increased serum glutamate levels in patients switched to CLZ [33].

CLZ is also posited to modulate various receptors, transporters, and enzymes involved in glutamate signaling, consequently implicating single nucleotide polymorphism (SNP) gene variants within these pathways as strong candidates for assessing susceptibility to CLZ nonresponse. In regard to glutamate receptors, CLZ administration increases α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor density and AMPA receptor subunit 1 (GluR1) expression [34, 35]. The GluR1 subunit protein is encoded by the *GRIA1* gene mapped to 5q31.1 [36]. CLZ also increases metabotropic glutamate receptor 2 (mGluR2) signaling [37], which is encoded by the *GRM2* gene mapped to 3p21.1 [38]. AMPA and mGluR glutamate receptors are involved in primary depolarization of glutamate-mediated neurotransmission and synaptic plasticity [39, 40].

With respect to transporter proteins, CLZ reportedly downregulates expression of glutamate transporter 1 (GLT1) in the rat cerebral cortex [41]. GLT1, encoded by the *SLC1A2* gene that maps to region 11p13–p12 [42], localizes to astrocytes in the mammalian CNS and is responsible for the greatest proportion of total glutamate reuptake from the synapse [43]. Functional inactivation of GLT1 raises extracellular glutamate levels, a biological phenomenon implicated in AP nonresponse [44]. CLZ also increases synaptic glycine concentrations [45]. In neuronal tissue, the glycine transporter 1 (GlyT1) chiefly determines the availability of glycine in the brain by mediating glycine reuptake into surrounding nerve terminals and glial cells [46]. The *SLC6A9* gene codes for GlyT1 and is mapped to 1p33 [47].

Lastly, CLZ has been shown to downregulate promoter methylation of the glutamate decarboxylase 1 (GAD1) enzyme in mice [48]. GAD1 catalyzes the decarboxylation of glutamic acid to the inhibitory neurotransmitter GABA (γ-aminobutyric acid) and carbon dioxide [49] and is encoded by the *GAD1* gene that maps to 2q.31 [50]. Interestingly, abnormal GABAergic function is also reported in patients with SCZ [51].

Based on the aforementioned body of evidence, the present study set out to investigate the contribution of glutamate variants distributed in the genes *SLC1A2*, *SLC6A9*, *GRIA1*, *GRM2*, and *GAD1* to CLZ response in a sample of subjects with TRS or treatment intolerance to other APs.

Subjects and Methods

Study Sample

Subjects included in this study were recruited from three clinical sites: Case Western Reserve University in Cleveland, Ohio (H.Y.M., n = 74); Hillside Hospital in Glen Oaks, N.Y. (J.A.L., n = 73), and University of California, Irvine, Calif. (S.G.P., n = 28) (total sample, n = 175). All patients had a diagnosis of SCZ or schizoaffective disorder according to the Diagnostic and Statistical Manual for Mental Disorders III-R or IV (DSM-III-R/IV) [52, 53] and met criteria for either TRS or intolerance to standard pharmacotherapy. TRS was defined as failure to respond to 2 or more AP

Table 1. Glutamate system gene variants genotyped in this study

Gene	SNP ID	Mn>Mj	Region position	Functional characterization	MAF	References
GRM2	rs4067	A>G	Promoter 51738256	HaploReg: EHM; NIEHS: TFBS, miRNA; transition substitution	0.163	MDD, BP, fluvoxamine response [77]; METH-IP, SCZ [78]
GRM2	rs2518461	A>G	Promoter 51738101	HaploReg: EHM, DNAse, MC; NIEHS: TFBS, miRNA; RegulomeDB score: 2a; transition substitution	0.071	NA
SLC1A2	rs4354668 (–181A>C)	G>T	5′ UTR Promoter 35440976	HaploReg: Cons, PHM, EHM, DNAse, PB, MC; NIEHS: TFBS; RegulomeDB score: 2b; transversion substitution	0.457	BP, lithium response [79]; SCZ [80]; gene expression [81]
SLC1A2	rs4534557	G>C	Intronic 35413964	HaploReg: Cons, PHM, EHM, DNAse, PB, MC; RegulomeDB Score: 2a; transversion substitution	0.396	SCZ [82, 83]
SLC1A2	rs2901534	C>G	Intronic 35364569	HaploReg: PHM, EHM, DNAse, PB, MC; transversion substitution	0.211	NA
SLC6A9	rs12037805	C>T	Intronic 44497249	HaploReg: Cons, PHM, EHM, DNAse, PB, MC; NIEHS: TFBS; transition substitution	0.339	NA
SLC6A9	rs1978195	G>A	2.1 kb upstream 44499242	HaploReg: PHM, EHM, DNAse, PB, MC; NIEHS: TFBS; transition substitution	0.407	NA
SLC6A9	rs16831558	T>C	5 kb upstream 44502163	HaploReg: EHM, DNAse, MC; RegulomeDB Score: 2b; transition substitution	0.123	NA
GRIA1	rs2195450	A>G	Intronic 152871009	HaploReg: Cons, PHM, EHM, MC; NIEHS: TFBS; transition substitution	0.239	NA
GAD1	rs3749034	A>G	5′ UTR exon 1 171673475	HaploReg: Cons, PHM, DNAse, PB, MC; NIEHS: TFBS; transition substitution	0.253	SCZ [84–86]

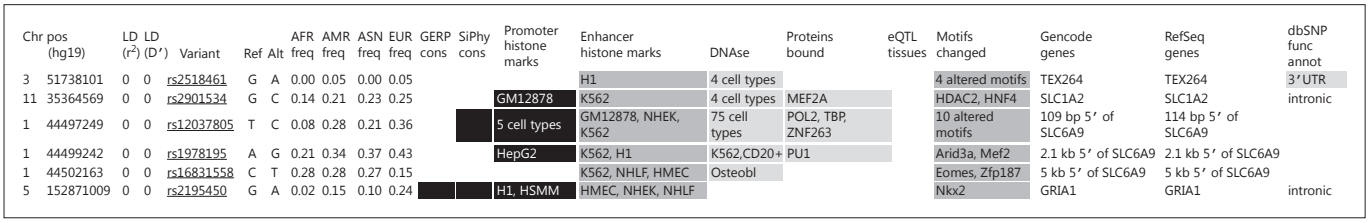
Mn>Mj = Minor>major allele; UTR = untranslated region; EHM = enhancer-associated histone mark; NIEHS = National Institute of Environmental Health Sciences; TFBS = transcription factor binding site; miRNA = microRNA binding site; MC = motifs changed; 2a/2b = likely to affect binding; Cons = evolutionarily conserved variant; PHM = promoter-associated histone mark; PB = protein bound; MAF = minor allele frequency in our sample (n = 163); BP = bipolar disorder; MDD = major depressive disorder; METH-IP = methamphetamine-induced psychosis; SCZ = schizophrenia; NA = not available.

trials with drugs from at least 2 different chemical classes at doses of ≥1,000 mg/day chlorpromazine equivalents for 4–6 weeks, together with no period of good functioning in the preceding 5 years [7]. A smaller portion of patients (<15%) met criteria for treatment intolerance defined as the presence of moderate-to-severe tardive dyskinesia or extreme sensitivity to extrapyramidal symptoms [18].

For the genetic studies, written informed consent was obtained from all study participants in accordance with the Ethical Principles for Medical Research Involving Human Subjects at the Centre for Addiction and Mental Health (CAMH) and with the Declaration of Helsinki as revised in 1989 [54]. Study participants underwent a 2- to 4-week washout period during which no medication was administered unless clinically necessary. During the washout period, benzodiazepines were the only class of drugs permissible in cases where treatment was clinically necessary; however, they were used sparingly. The patients were then placed on CLZ monotherapy for 6 months or longer with mean doses in the range of 450 mg/day. Benzodiazepines were the only class of drugs administered intermittently during the titration period. For the duration of the study, the patients were seen weekly for blood draws to monitor white blood cell/neutrophil counts as a precaution against CLZ-induced agranulocytosis.

Response Measures

CLZ response was assessed using both categorical and continuous response measures. Categorical response was assessed using change in Brief Psychiatric Rating Scale (BPRS) scores between baseline and following 6 months of CLZ therapy. Participants were considered responders if they experienced a ≥20% decrease in



Chr	pos (hg19)	LD (r^2)	LD (D′)	Variant	Ref	Alt	AFR freq	AMR freq	ASN freq	EUR freq	GERP cons	SiPhy cons	Promoter histone marks	Enhancer histone marks	DNAse	Proteins bound	eQTL tissues	Motifs changed	Gencode genes	RefSeq genes	dbSNP func annot
3	51738101	0	0	rs2518461	G	A	0.00	0.05	0.00	0.05				H1	4 cell types			4 altered motifs	TEX264	TEX264	3′UTR
11	35364569	0	0	rs2901534	G	C	0.14	0.21	0.23	0.25			GM12878	K562	4 cell types	MEF2A		HDAC2, HNF4	SLC1A2	SLC1A2	intronic
1	44497249	0	0	rs12037805	T	C	0.08	0.28	0.21	0.36			5 cell types	GM12878, NHEK, K562	75 cell types	POL2, TBP, ZNF263		10 altered motifs	109 bp 5′ of SLC6A9	114 bp 5′ of SLC6A9	
1	44499242	0	0	rs1978195	A	G	0.21	0.34	0.37	0.43			HepG2	K562, H1	K562,CD20+	PU1		Arid3a, Mef2	2.1 kb 5′ of SLC6A9	2.1 kb 5′ of SLC6A9	
1	44502163	0	0	rs16831558	C	T	0.28	0.28	0.27	0.15				K562, NHLF, HMEC	Osteobl			Eomes, Zfp187	5 kb 5′ of SLC6A9	5 kb 5′ of SLC6A9	
5	152871009	0	0	rs2195450	G	A	0.02	0.15	0.10	0.24			H1, HSMM	HMEC, NHEK, NHLF				Nkx2	GRIA1	GRIA1	intronic

Fig. 1. HaploReg database results for 6 putatively functional variants included in this study [55]. To be considered for study inclusion, variants were required to have a minor allele frequency of ≥5% and to satisfy ≥3 of 7 functionality criteria. Chr = Chromosome; pos = position; LD = linkage disequilibrium; r^2 = correlation coefficient; D′ = Hedrick's multiallelic D′; Ref = reference allele; Alt = alternate allele; AFR/AMR/ASN/EUR freq = African/admixed American/Asian/European minor allele frequency; GERP/SiPhy cons = genomic evolutionary rate profiling/SiPhy mammalian conservation algorithms; DNAse = DNAse I hypersensitivity site; eQTL = expression quantitative trait loci; dbSNP func annot = dbSNP functional annotation.

BPRS scores. Continuous response was measured as percent score reduction for the total BPRS and for the positive (BPOS) and negative (BNEG) subscales, using the following calculation: [(6-month score – baseline score)/baseline score]. Using this formula, scores below 0 indicate symptom improvement.

SNP Selection

In total, 10 glutamate gene variants were included in our study (table 1). Four variants were selected based on previous investigation into other neuropsychiatric phenotypes as cited in the literature, and the remaining 6 were selected from several functional annotation websites based on potential to alter gene expression.

Identification of SNPs from the Literature. A literature search was conducted on PubMed using the following search terms: *'GRM2'*, 'Metabotropic Glutamate Receptor 2', *'SLC1A2'*, 'Glutamate Transporter 1', 'Excitatory Amino Acid Transporter 2', 'EAAT2', *'SLC6A9'*, 'Glycine Transporter 1', 'AMPA', *'GRIA1'*, 'GluA1', *'GAD1'*, and 'Glutamate Decarboxylase 1'. Articles investigating genetic associations (genome-wide and candidate SNP studies) with neuropsychiatric phenotypes were considered. In addition, articles investigating the functional significance (positron emission tomography ligand binding assays, electrophoretic mobility shift assays, luciferase promoter assays, etc.) of variants within *SLC1A2*, *SLC6A9*, *GRIA1*, *GRM2*, and *GAD1* were reviewed to identify glutamate system SNPs of interest.

Identification of SNPs with Potential Functionality. The Broad Institute's HaploReg database (http://www.broadinstitute.org/mammals/haploreg/haploreg.php) was used to identify glutamate variants with potentially functional effects on gene expression (fig. 1) [55]. The genes' coding regions, plus an additional 10 kb upstream and 2 kb downstream, were submitted to the 'Build Query' search field on HaploReg. Gene regions (chrN:start-end) were obtained from the UCSC (University of California Santa Cruz) Genome Browser using Human Genome assembly version 19: *GRM2* (chr3:51,731,081–51,754,625), *SLC1A2* (chr11:35,270,752–35,461,610), *SLC6A9* (chr1:44,455,172–44,507,164), and *GRIA1* (chr5:152,849,175–153,088,732). Seven criteria for measuring functionality were provided for each SNP: (1) sequence conservation across mammals, (2) promoter histone marks, (3) enhancer histone marks, (4) DNAse hypersensitivity sites, (5) bound proteins, (6) expression quantitative trait locus data, and (7) the effect on regulatory motifs. SNPs with a minor allele frequency of ≥5% in the European population that satisfied ≥3 of the 7 functional criteria were considered for inclusion. Six variants satisfying these criteria were selected: *SLC1A2* (rs2901534), *SLC6A9* (rs12037805, rs1978195, and rs16831558), *GRIA1* (rs2195450), and *GRM2* (rs2518461) (fig. 2). RegulomeDB [56] and the National Institute of Environmental Health Sciences (NIEHS) Functional SNP Prediction (FuncPred) database scores [57] were also obtained to further assess functionality.

DNA Isolation and Genotyping

Venous blood samples were collected from the study participants and sent to our center (CAMH, Toronto, Ont., Canada), where genomic DNA was extracted using the high salt method [58]. Genotyping was performed using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, Calif., USA). The final PCR reaction mixtures consisted of 20 ng of genomic DNA and 2 μl of 2× TaqMan® OpenArray® Genotyping Master Mix. Samples were loaded onto a QuantStudio Digital PCR Plate using the QuantStudio 12K Flex AccuFill System and run on the QuantStudio 12K Flex Instrument as per the manufacturer's instructions. Genotype calls were visualized using the DigitalSuite Software and were validated by two independent researchers blinded to the genotyping conditions. To ensure genotyping accuracy, 10% of the samples were re-genotyped, and conflicting genotypes were set as missing for future analyses.

Statistical Analyses

Quality control (QC) of the genotyping data was carried out using PLINK v1.07 [59]. In order to pass QC, SNPs were required to have a ≥90% genotyping rate, to have a minor allele frequency of ≥0.05, and to satisfy the Hardy-Weinberg equilibrium ($p > 0.005$). In addition, individuals with a genotyping efficiency <80% across all markers (samples producing genotypes for ≤7 SNPs) were excluded from analysis.

Descriptive statistics were carried out using the Statistical Package for the Social Sciences (SPSS) v20 (IBM Corporation, Armonk, N.Y., USA). Continuous variables were analyzed using Student's

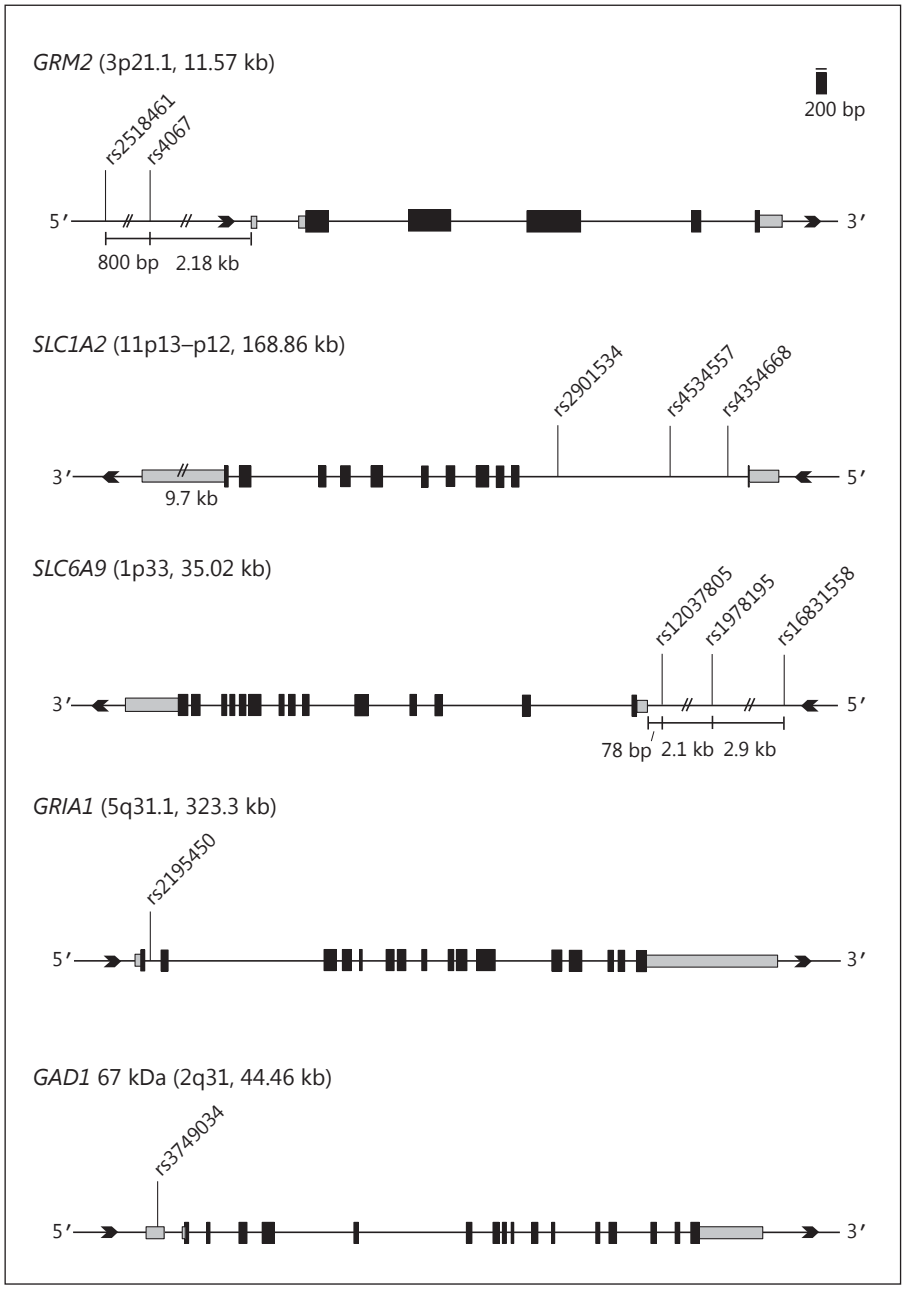


Fig. 2. Chromosome location, size, and schematic representation of the *GRM2*, *SLC1A2*, *SLC6A9*, *GRIA1*, and *GAD1* genes. The SNPs genotyped in this study are shown. Dark boxes represent coding exons and light boxes represent 5′ and 3′ untranslated regions. The gene diagrams were constructed using the NCBI Reference Sequence from *Homo sapiens*, transcript variant 1. Alternatively spliced variants exist for all 5 genes and can be viewed on NCBI AceView [64].

t test or analysis of variance (ANOVA), and categorical variables were analyzed using Pearson's χ^2 test. Genotype and allele frequencies in the responder/nonresponder groups were compared using the χ^2 test, or by Fisher's exact test for cell counts <5. Differences in percent score reductions in the BPRS and the BPOS and BNEG subscales among genotype groups were compared using analysis of covariance (ANCOVA), with age and baseline scores added as covariates.

The Hardy-Weinberg equilibrium and the linkage disequilibrium (LD) among SNPs were determined using Haploview v4.2 and the Solid Spine of LD to construct LD blocks (fig. 3) [60]. Haplotype analyses were carried out using UNPHASED v3.1.5, and haplotype p values were corrected for multiple testing using permutation (10,000 tests) [61]. Haplotypes with a frequency <0.05 were removed from subsequent analyses. Power calculations were performed using Quanto v1.2.4 [62]. Multiple testing correction was performed using the Nyholt method and resulted in a revised statistical significance threshold of $p < 0.005$ [63].

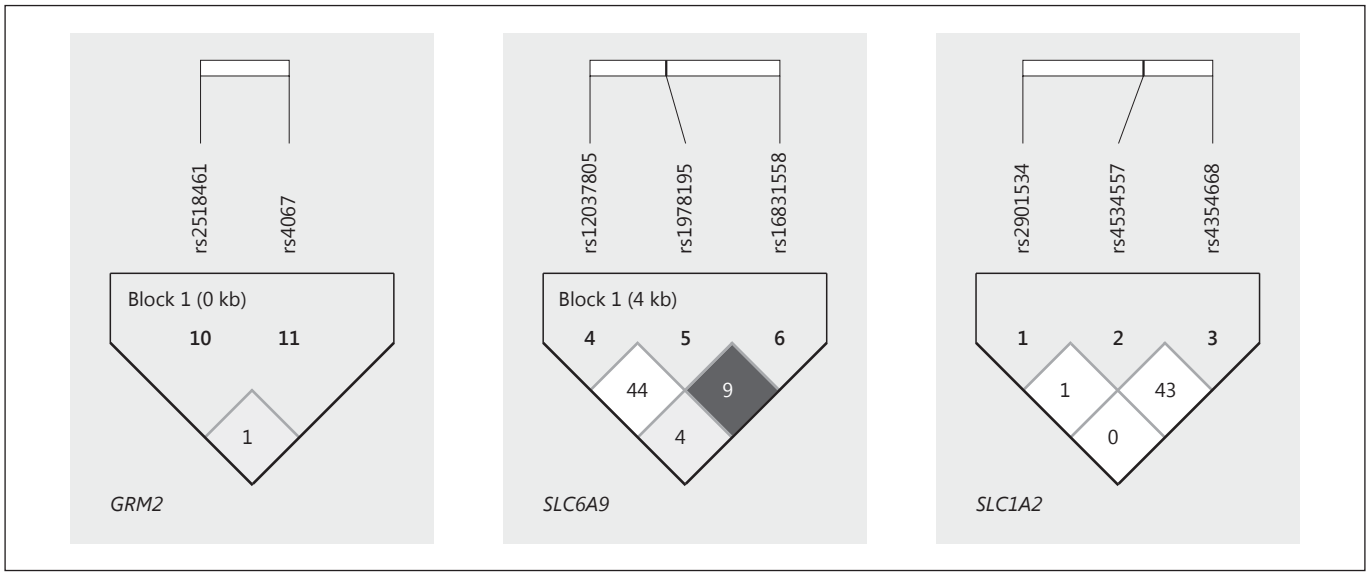


Fig. 3. *GRM2*, *SLC6A9*, and *SLC1A2* LD plots (Haploview v4.2). Haplotype blocks defined using Solid Spine of LD. The confidence bounds color scheme is used. Dark gray: strong evidence of LD; light gray: uninformative; white: strong evidence of recombination. Correlation coefficient values as percentages (r^2) are displayed within each box.

Table 2. Demographics and clinical information for the study samples

	H.Y.M. (n = 66)	J.A.L. (n = 69)	S.G.P. (n = 28)	p value	Total (n = 163)
Age, years	32.70±7.78	35.70±8.27	39.15±12.78	0.001[b]	35.07±8.10
Gender				0.150[c]	
Male	48 (72.7)	49 (71.0)	25 (89.3)		122 (74.8)
Female	18 (27.3)	20 (29.0)	3 (10.7)		41 (25.2)
R/NR[a]	33/33 (50.0/50.0)	40/29 (58.0/42.0)	13/15 (46.4/53.6)	0.496[c]	86/77 (52.8/47.2)
ΔBPRS scores (n = 94)	−10.05±12.67	NA	−6.82±11.78	0.253[b]	−9.09±12.44
ΔBNEG scores (n = 93)	−1.32±3.40	NA	−0.57±4.19	0.365[b]	−1.10±3.65
ΔBPOS scores (n = 91)	−3.13±5.30	NA	−3.25±6.65	0.925[b]	−3.16±5.71

Values are presented as means ± SD or n (%). SD = Standard deviation; R/NR = responders/nonresponders; BPRS = Brief Psychiatric Rating Scale; BNEG/BPOS = BPRS negative and positive subscales; NA = data not available. [a] Responders defined as having a ≥20% reduction in BPRS scores from baseline. [b] p values from Student's t test or one-way ANOVA. [c] p values from χ^2 test.

Results

Study Sample Characteristics

Following the QC procedures, 12 individuals were removed due to low genotyping efficiency (<80% of the markers were successfully genotyped). The remaining sample had a mean genotyping efficiency of 98.9%. All SNPs met the QC criteria and had a mean genotyping rate of 99.3%. In total, 163 European treatment-resistant/-intolerant patients were included in the analyses (table 2). Our sample had >80% power to detect an odds ratio as low as 2.1 (unmatched case-control design, n = 163; nonresponder frequency = 47.2; minor allele frequency = 26.5%; α/2 = 0.05) and down to 8.5% of the variance in the quantitative response variable (continuous design, n = 91). The average minor allele frequency across the 10 glutamate variants was used for power calculations.

Table 3. Association analysis among glutamate system gene variants and CLZ response

Gene SNP	HWE	Genotype	R/NR[a]	ΔBPRS±SD[b]	ΔBPOS±SD[b]	ΔBNEG±SD[b]	Allele	R/NR[a]
GRM2	0.7838	AA	1/4	–6.67±12.66	0.67±8.14	–3.00±4.36	A	26/30
rs4067		AG	23/22	–8.14±10.78	–2.57±6.72	–1.14±3.91		
		GG	62/51	–9.49±13.05	–3.52±5.27	–1.00±3.58	G	148/124
		p	0.497[c]	0.666	0.223	0.688	p	0.276
GRM2	0.6025	AA	1/0	NA	NA	NA	A	12/12
rs2518461		AG	10/12	–11.30±12.53	–6.70±3.77	0.30±3.62		
		GG	75/65	–8.86±12.55	–2.76±5.80	–1.26±3.66	G	162/142
		p	0.657[c]	0.535	0.335	**0.037**	p	0.756
SLC1A2	0.7605	GG	17/14	–11.29±11.61	–3.56±5.54	–1.25±3.30	G	80/67
rs4354668		GT	44/39	–7.57±11.85	–3.02±5.17	–0.17±3.13		
		TT	25/24	–10.94±13.58	–3.70±6.69	–2.19±4.35	T	94/87
		p	0.944	0.430	0.912	**0.026**	p	0.653
SLC1A2	0.1155	GG	17/11	–12.24±10.82	–2.60±4.43	–2.00±3.16	G	69/57
rs4534557		CG	33/35	–6.63±11.26	–3.91±5.82	0.20±2.88		
		CC	35/31	–9.50±14.29	–2.51±6.14	–1.86±4.31	C	103/97
		p	0.548	0.212	0.834	**0.015***	p	0.566
SLC1A2	0.8233	CC	5/1	–16.00±8.88	–3.80±4.32	–4.33±3.98	C	39/28
rs2901534		CG	29/26	–10.12±12.21	–3.71±5.69	–1.71±3.24		
		GG	52/50	–7.70±12.88	–2.80±5.93	–0.31±3.68	G	135/126
		p	0.628[c]	0.343	0.551	**0.047**	p	0.343
SLC6A9	0.1462	TT	2/3	5.67±4.04	3.00±1.73	2.67±1.53	T	22/21
rs16831558		TC	18/15	–7.27±11.37	0.00±5.66	–2.13±3.83		
		CC	66/59	–10.41±12.37	–4.25±5.42	–1.07±3.63	C	152/133
		p	1.00[c]	**0.045**	**0.008**	0.104	p	0.790
SLC6A9	0.1782	CC	14/8	–10.77±9.55	–3.50±4.77	–1.86±3.70	C	64/43
rs12037805		CT	36/27	–12.09±12.95	–4.19±5.39	–1.48±4.23		
		TT	35/42	–6.52±12.79	–2.39±6.27	–0.62±3.27	T	108/111
		p	0.203	0.238	0.663	0.198	p	0.0746
SLC6A9	0.7521	GG	17/8	–10.27±9.27	–2.00±4.761	–1.75±4.31	G	73/57
rs1978195		AG	38/41	–10.74±12.36	–5.07±5.69	–1.10±3.45		
		AA	30/26	–6.39±14.46	–1.23±5.86	–0.71±3.81	A	99/93
		p	0.213	0.445	0.143	0.179	p	0.418
GRIA1	0.4035	AA	8/4	–19.50±18.27	–6.50±2.89	–4.00±5.48	A	46/36
rs2195450		AG	30/28	–8.70±11.92	–3.45±5.81	–1.26±3.19		
		GG	48/44	–8.42±11.91	–2.81±5.88	–0.65±3.66	G	128/116
		p	0.874[c]	0.174	0.664	**0.017**	p	0.568
GAD1	0.6878	AA	5/6	–6.75±9.54	–3.57±6.02	–2.00±3.93	A	44/36
rs3749034		AG	34/24	–10.47±13.95	–3.33±6.16	–1.70±4.33		
		GG	44/47	–8.33	–3.04±5.74	–0.26±2.86	G	124/118
		p	0.428	0.794	0.986	0.349	p	0.559

HWE = Hardy-Weinberg equilibrium; R/NR = responders/nonresponders; BPRS = Brief Psychiatric Rating Scale; BNEG/BPOS = BPRS negative and positive subscale; SD = standard deviation. [a] p values from χ^2 test. [b] p values from ANCOVA with baseline scores and age added as covariates. [c] p values from Fisher's exact test; category with low cell value (<5) collapsed with heterozygous group. * p value from Welch's ANOVA test due to a significant Levine test result. Boldfaced p values indicate nominally significant findings obtained prior to correction (<0.05).

Table 4. Haplotype analysis for *GRM2* and CLZ response

Haplotype[a]	rs4067-rs2518461			
	R/NR p = 0.711[b]	ΔBPRS p = 0.719[b]	ΔBPOS p = 0.190[b]	ΔBNEG p = 0.133[b]
A-G	27/30 (15.0/18.1) p = 0.441	28 (16.3) p = 0.695	27 (15.9) p = 0.132	27 (15.7) p = 0.567
G-A	12/12 (6.7/7.2) p = 0.837	10 (5.8) p = 0.506	10 (5.9) p = 0.236	10 (5.8) p = 0.055
G-G	141/124 (78.3/74.7) p = 0.425	134 (77.9) p = 0.456	133 (78.2) p = 0.517	135 (78.5) p = 0.541

Values are presented as n (%). R/NR = Responders/nonresponders; BPRS = Brief Psychiatric Rating Scale; BNEG/BPOS = BPRS negative and positive subscales. [a] Haplotypes with a frequency <0.05 were excluded. [b] Uncorrected p values from UNPHASED v3.1.5.

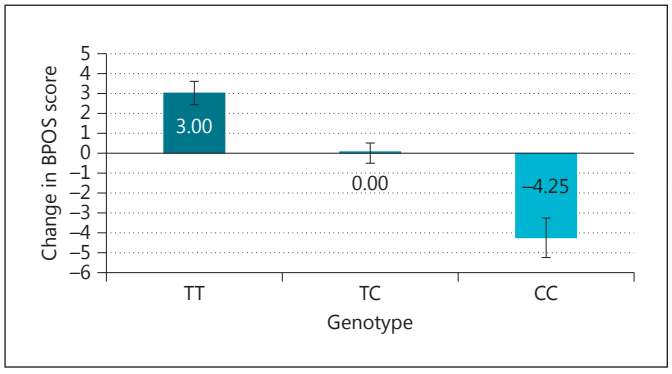


Fig. 4. C allele carriers of the *SLC6A9* variant rs16831558 experienced an allele dose-dependent reduction in BPOS scores following 6 months of CLZ therapy ($p_{uncorrected}$ = 0.008, $p_{corrected}$ = 0.08, assuming 9.36 independent tests); however, this finding did not remain significant following correction for multiple testing.

Among the three clinical sites, no statistically significant differences were observed for gender, response rates, or percent score reductions in the BPRS, BPOS, and BNEG scales. A statistically significant difference was observed, however, for age of the study participants across the clinical sites (p = 0.001). Post hoc analysis using Scheffé's test for unequal sample sizes revealed that patients originating from S.G.P.'s sample were significantly older than patients from H.Y.M.'s sample. Baseline BPRS, BPOS, and BNEG values were also correlated with their respective percent reduction scores. In order to combine the three samples for analysis, both age and baseline scores were included as covariates in the analyses of continuous response. The combined sample (n = 163) consisted of 74.8% males and had a mean age of 35.1 years (SD 8.1). Following 6 months of CLZ therapy, the patients experienced a mean percent reduction of –9.1 (SD 12.4), –1.1 (SD 3.6), and –3.2 (SD 5.7) from baseline for the BPRS, BNEG, and BPOS scales, respectively. In addition, 52.8% of the patients experienced a ≥20% decrease in BPRS scores and were subsequently classified as CLZ responders.

Glutamate System Variant Genotype and Allele Association Analyses

Following correction for multiple testing, no significant differences between the responder and nonresponder groups or as measured by change in percent scores using the BPRS, BPOS, and BNEG scales were observed for genotype and allele frequencies for each of the 10 glutamate system gene variants (table 3). A number of nominally significant associations were observed prior to correction; however, none were significant following correction for multiple testing.

SLC6A9 rs16831558 C allele carriers experienced an allele dose-dependent reduction (improvement) in BPOS subscale scores following 6 months of CLZ therapy ($p_{uncorrected}$ = 0.008, $p_{corrected}$ = 0.08) (fig. 4). CC homozygotes of rs16831558 also experienced a greater reduction in BPRS total scores ($p_{uncorrected}$ = 0.045, $p_{corrected}$ = 0.421). In addition, greater reductions in BNEG subscale scores were observed for 5 variants: *SLC1A2* rs4534557 homozygotes (GG and CC; $p_{uncorrected}$ = 0.015, $p_{corrected}$ = 0.140), *GRIA1* rs2195450 A allele carriers ($p_{uncorrected}$ = 0.017, $p_{corrected}$ = 0.162), *SLC1A2* rs4354668 TT homozygotes

Table 5. Haplotype analysis for *SLC6A9* and CLZ response

Haplotype[a]	rs16831558-rs12037805-rs1978195				rs12037805-rs1978195[b]
	R/NR p = 0.159[c]	ΔBPRS p = 0.020[c]	ΔBNEG p = 0.096[c]	ΔBPOS p = 0.001[c]	ΔBPOS p = 0.757[c]
C-C-G	54/35 (34.2/24.0) p = 0.051	44 (28.6) p = 0.066	45 (29.2) p = 0.022	45 (29.6) p = 0.781	45 (29.6) p = 0.614
C-T-A	70/67 (44.3/45.9) p = 0.781	71 (46.1) p = 0.404	70 (45.4) p = 0.530	68 (44.7) p = 0.079	68 (44.7) p = 0.483
C-T-G	16/23 (10.1/15.7) p = 0.143	21 (13.6) p = 0.167	20 (13.0) p = 0.551	20 (13.2) p = 0.905	20 (13.2) p = 0.730
T-T-A	18/21 (11.4/14.4) p = 0.436	18 (11.7) p = 0.016	19 (12.3) p = 0.143	19 (12.5) p = 0.001	19 (12.5) p = 1.000

Values are presented as n (%). R/NR = Responders/nonresponders; BPRS = Brief Psychiatric Rating Scale; BNEG/BPOS = BPRS negative and positive subscales. [a] Haplotypes with a frequency <0.05 were excluded. [b] rs16831558 included as 'conditioning marker' to two-marker haplotype. [c] Uncorrected p values from UNPHASED v3.1.5.

($p_{uncorrected}$ = 0.026, $p_{corrected}$ = 0.243), *GRM2* rs2518461 GG homozygotes ($p_{uncorrected}$ = 0.037, $p_{corrected}$ = 0.346), and *SLC1A2* rs2901534 CC homozygotes ($p_{uncorrected}$ = 0.047, $p_{corrected}$ = 0.440).

GRM2 and SLC6A9 Haplotype Analyses

No haplotype blocks within *GRM2* rs4067-rs2518461 were significantly different between responder/nonresponder groups, or in regard to continuous response measures (table 4). Initially, the haplotype block rs16831558-rs12037805-rs1978195 T-T-A within *SLC6A9* appeared significantly associated with change in BPOS scores following CLZ treatment ($p_{uncorrected}$ = 0.001, $p_{corrected}$ = 0.006, permutation corrected, individual haplotype effect). However, further investigation revealed that the positive association was driven solely by the rs16831558 marker; the *SLC6A9* rs12037805-rs1978195 haplotype, when analyzed independently with the rs16831558 variant added as a conditioning marker, was no longer significant (table 5).

Discussion

This investigation explored the association between 10 glutamate system gene variants and clinical response to CLZ. No significant associations were observed following correction for multiple testing in our sample of patients. To our knowledge, though, this is the first reported study to investigate the role of *SLC1A2* (rs4354668, rs4534557, and rs2901534), *SLC6A9* (rs12037805, rs1978195, and rs16831558), *GRIA1* (rs2195450), *GRM2* (rs4067 and rs2518461), and *GAD1* (rs3749034) in response to CLZ. Four of these variants had previously been investigated in other neuropsychiatric phenotypes, while the remaining 6 were previously unstudied SNPs selected using HaploReg.

Several nominally significant associations of *SLC6A9*, *SLC1A2*, *GRM2*, and *GRIA1* with CLZ response were observed before correcting for the number of independent tests. Of interest was the association between C allele carriers of *SLC6A9* rs16831558 and percent score reduction in the BPOS subscale ($p_{uncorrected}$ = 0.008). Individuals carrying two copies of the C allele showed a 4.25% decrease in severity of positive symptoms, while those carrying one copy experienced no symptom change, and individuals with two copies of the T allele experienced worsening of their positive symptoms, as indicated by BPOS scores that were approximately 3.00% higher than the scores obtained at baseline. This finding became nonsignificant following correction ($p_{corrected}$ = 0.08), but given our relatively small sample size, this nominally significant finding for *SLC6A9* rs16831558 may deserve further investigation in larger CLZ response samples.

The *SLC6A9* locus is particularly complex, with 16 different mRNA transcripts and 14 different splice variants [64]. The rs16831558 variant reported herein is located approximately 5 kb upstream of the *SLC6A9* start site.

This variant has a RegulomeDB [56] functional score of 2b ('likely to affect binding') and is predicted to lie within an enhancer histone mark, a DNAse I hypersensitivity site, and to affect regulatory motifs [55]. Therefore, characterization of this SNP's potential functionality using mobility shift and luciferase promoter assays may be a priority for future work.

GlyT1 has recently been the focus of several novel therapies for SCZ [65]. One of the most developmentally advanced drugs is bitopertin (RG1678), a synthetic GlyT1 inhibitor developed by Roche. Five phase III clinical trials have been conducted to investigate bitopertin as an adjunct to conventional AP therapy for the treatment of persistent negative symptoms or partial responders, although the results are nonsignificant (ClinicalTrials.gov identifiers:NCT01192867,NCT01192906,NCT01192880, NCT01235520, and NCT01235559). Biologically, GlyT1 regulates extracellular glycine concentrations in the vicinity of excitatory glutamatergic synapses [66]. Glycine, in turn, acts as a necessary co-agonist at the NR1 subunit and promotes NMDAR function [67, 68]. GlyT1 inhibitors block glycine reuptake from the synapse, causing an increase in NR1 glycine site occupancy and NMDAR potentiation, thus ameliorating the NMDAR hypofunction thought to contribute to SCZ etiology.

Following the same rationale, various NR1 glycine site agonists including glycine, D-serine, and D-cycloserine have been investigated as adjuncts to conventional AP therapy for treating persistent negative and cognitive symptoms in SCZ [reviewed in 69]. Even though glycine agonist drugs are not designed to improve the positive symptoms seen in TRS patients, observations of their use in clinical studies to improve negative symptoms have shed light on a possible action of CLZ on the glycine system: NR1 glycine agonists are generally well received when paired with most APs, but they provide little therapeutic benefit and may in fact worsen symptoms when administered to patients taking CLZ [23, 70]. This incompatibility between NR1 agonists and CLZ may be due to CLZ's preexisting ability to potentiate NMDAR-mediated neurotransmission through an as yet unknown mechanism [71]. Several mechanisms have been posited; for instance, CLZ may already increase synaptic glycine levels [45] or may act as a partial agonist at the NR1 glycine site of the NMDAR [72]. Alternatively, CLZ's ability to alter dopamine activity and achieve therapeutic effects may depend in part on the availability of the NMDAR glycine site [73]. Future studies are necessary to clarify the role of glycine receptor gene variants in the mechanism of CLZ's action.

There are some limitations inherent in our study that deserve to be mentioned. Our small sample size may not have had sufficient statistical power to detect the effect sizes of these gene variants, and going forward the collection of larger samples will help determine the contribution of genetics to the phenotype of CLZ response. One such sample is currently being collected by the CRESTAR consortium in Europe [74] with the goal of identifying markers involved in CLZ response and side effects. Heterogeneity within our study sample may also have confounded association findings. The participants were collected from three clinical sites that may have differed in population substructure. In addition, the study inclusion criteria were relatively broad; for example, patients with a diagnosis of either SCZ or schizoaffective disorder were eligible for study inclusion.

Incomplete outcome data may also have limited our findings. Individuals who quit prior to study completion invariably affect response rates and may confound the ability to identify genetic variants linked to treatment response. Unfortunately, rates for treatment discontinuation in AP drug trials are estimated to be in the range of 40%, with common reasons for discontinuation including noncompliance, lack of efficacy, and adverse side effects [18, 75, 76]. In certain patients, response to CLZ may take up to 5 months or longer, suggesting that some participants who discontinue treatment because of lack of efficacy most likely do so prematurely. The high rates of discontinuation during AP drug trials stress the need for understanding the interindividual variability in drug response phenotypes and emphasize the utility associated with identifying clinically predictive markers.

To conclude, we report no significant associations between 10 glutamate system gene variants and response to CLZ in our sample following correction for multiple testing. A nominally significant association with the GlyT1 gene variant rs16831558 was observed prior to correction, and may deserve further examination in larger well-characterized CLZ response samples. Future work may consider investigating the potential for other gene variants in the glutamate system to alter response to CLZ, as well as the genetic effect of these variants on additional symptom domains such as cognition.

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Author Contributions

J.L.K., D.L.T., D.J.M., J.K., and G.R. designed the study. H.Y.M., J.A.L., and S.G.P. provided the samples. D.L.T. collected the genotype data and managed the literature searches and analyses. D.L.T. and A.K.T. undertook the statistical analysis. D.L.T. wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Statement of Ethics

Written informed consent was obtained from all study participants in accordance with the Ethical Principles for Medical Research Involving Human Subjects at the Centre for Addiction and Mental Health (CAMH) and with the Declaration of Helsinki, as revised in 1989.

Disclosure Statement

D.L.T., A.K.T., D.J.M., and J.K. report no competing interests. J.A.L. serves on the Advisory Board of Bioline, Intracellular Therapies, and PsychoGenics and receives no direct financial compensation or salary support for participation in research, consulting, or advisory board activities. J.A.L. receives grant support from Allon, La Roche, GlaxoSmithKline, Eli Lilly, Merck, Novartis, Pfizer, PsychoGenics, Sepracor (Sunovion), and Targacept, and he holds a patent from Repligen. S.G.P. has received grant support funding, honoraria, and/or a paid consultantship from – or served as a consultant/advisory board member to – Alkermes, AstraZeneca, Bioline, Bristol-Myers Squibb, Ceregene, Cephalon, Sunovion, Elan, Eisai, Eli Lilly, Forest Laboratories, Fujisawa Healthcare, Genentech, Janssen Pharmaceutica, Merck, Novartis, Ono, Organon, Otsuka, Pfizer, Solvay Pharmaceuticals, Lundbeck, La Roche, NIH, Harvard-Massachusetts General Hospital, Brigham and Women's Hospital, Vanda Pharmaceuticals Inc., Cortex, Schering Plough, and Takeda. H.Y.M. has received grants from – or is a consultant to – Abbott Labs, ACADIA, Alkermes, Bristol-Myers Squibb, Dainippon Sumitomo, Eli Lilly, EnVivo, Janssen, Otsuka, Pfizer, La Roche, Sunovion, AstraZeneca, GlaxoSmithKline, and BioLineRx. H.Y.M. is also a shareholder of ACADIA and GlaxoSmithKline. G.R. has received research support from Novartis Canada, Medicure Inc., and Neurocrine, consultant fees from CanAm Bioresearch Inc., Neurocrine, and Medicure Inc., and speaker's fees from Novartis. J.L.K. has been a consultant to GlaxoSmithKline, Sanofi-Aventis, and Dainippon Sumitomo and has received honoraria from Novartis and Eli Lilly.

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