Brazilian Journal of Psychiatry
bjp
Revista Brasileira de Psiquiatria

Braz J Psychiatry. 2023 Mar-Apr;45(2):117-126
doi:10.47626/1516-4446-2022-2783
Brazilian Psychiatric Association

ORIGINAL ARTICLE

Influence of glutathione-related genetic variants on the oxidative stress profile of Mexican patients with psychotic disorders

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Objective: The clinical trajectories of patients with psychotic disorders have divergent outcomes, which may result in part from glutathione (GSH)-related high-risk genotypes. We aimed to determine pharmacokinetics of clozapine, GSH levels, GSH peroxidase (GPx) activity, gene variants involved in the synthesis and metabolism of GSH, and their association with psychotic disorders in Mexican patients on clozapine monotherapy and controls.
Methods: The sample included 75 patients with psychotic disorders on clozapine therapy and 40 paired healthy controls. Plasma clozapine/N-desmethylclozapine, GSH concentrations, and GPx activity were determined, along with genotyping of GCLC and GSTP1 variants and copy number variations of GSTP1, GSTT1, and GSTM1. Clinical, molecular and biochemical data were analyzed with a logistic regression model.
Results: GSH levels were significantly reduced and, conversely, GPx activity was higher among patients than controls. GCLC_GAG-7/9 genotype (OR = 4.3, 95%CI = 1.40-14.31, p = 0.019) and hetero-/homozygous genotypes of GCLC_rs761142 (OR = 6.09, 95%CI = 1.93-22.59, p = 0.003) were found to be risk factors for psychosis. The genetic variants were not related to clozapine/N-desmethylclozapine levels or metabolic ratio.
Conclusions: GCLC variants were associated with the oxidative stress profile of patients with psychotic disorders, raising opportunities for intervention to improve their antioxidant defenses. Further studies with larger samples should explore this proposal.

Keywords: Psychotic disorders; GCLC gene; glutathione; glutathione peroxidase activity; glutathione S-transferases

Introduction

Psychotic disorders (PD) are severe mental illnesses that cause abnormal thinking and perceptions. Two of the main symptoms are delusions and hallucinations; typical onset occurs in late adolescence or early adulthood.[1] There is growing evidence that redox dysregulation and its consequent oxidative stress are implicated in the pathophysiology of PD, including schizophrenia (SZ),[2] bipolar disorder (BD),[3] schizoaffective disorder (SD),[4] and interictal psychosis (IIP; also known as SZ-like psychosis in epilepsy).[5,6] Genetic and environmental factors converge on the redox system, contributing to the pathophysiology of psychosis. Environmental insults combined with a genetic susceptibility to redox dysregulation (e.g., variants in antioxidant defense-related genes) could be implicated through redox-sensitive and oxidative stress-mediated mechanisms.[7-9] In this context, it has been shown that patients with PD have low glutathione (GSH) levels in peripheral tissues,[10-13] in postmortem brain studies,[11,14,15] and in neuroimaging analysis of first-episode psychotic patients.[16] It has been suggested that decreased GSH levels may occur due to gene dosage effects between copy number variations (CNV) and reduced enzyme activity in GSH S-transferases (GSTs).[15,17] In addition, several genetic variants of the catalytic subunit

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Submitted Jul 16 2022, accepted Sep 27 2022.

How to cite this article: Mayén-Lobo YG, Alcaraz-Zubeldia M, Dávila-Ortiz de Montellano DJ, Motilla-Frías BA, García-Manteca MY, Ortega-Vázquez A, et al. Influence of glutathione-related genetic variants on the oxidative stress profile of Mexican patients with psychotic disorders. Braz J Psychiatry. 2023;45:117-126. http://doi.org/10.47626/1516-4446-2022-2783

of glutamate-cysteine ligase (GCLC) (rate-limiting synthesizing enzyme of GSH) have strong functional effects on GSH levels in fibroblasts when challenged with oxidative stress,[18] which has also been observed in brain studies.[16,18-20] Interestingly, Xin et al.[16] found a negative correlation between GSH peroxidase (GPx) activity and GSH levels in male early psychosis patients, which suggests that blood GPx activity may reflect central oxidative status.

On the other hand, evidence has shown the therapeutic potential of second-generation antipsychotics, particularly clozapine, for improving antioxidant status and reducing lipid peroxidation in patients with SZ and for modulating microglia activation and oxidative stress.[21] Currently, the link between oxidative stress, clinical response to clozapine, and its potential antioxidant properties is not fully understood, but it has been proposed that its ability to decrease neutrophil ROS production may depend on the amino group part of its chemical structure.[21]

In this study we aimed to determine plasma clozapine concentrations, metabolic ratio, GSH levels, GPx activity, and variants of genes encoding the main enzymes involved in the synthesis and metabolism of GSH (*GCLC* and *GSTs*) and their potential association with PD in Mexican patients on clozapine monotherapy and a paired healthy control group. We hypothesized that genetic variants and environmental factors could be modulating GSH concentrations/GPx activity and may be associated with the oxidative stress profile and clinical outcome of patients with PD who are clozapine responders (Figure 1).

Methods

Subjects

Unrelated Mexican Mestizo patients with a clinically diagnosed PD (SZ, SD, BD, and IIP) were recruited for this study. All patients had been taking oral clozapine as antipsychotic monotherapy for at least 6 months prior to enrollment. Clinical diagnosis of a PD according to the DSM-5 was determined by at least one neuropsychiatrist.[1] The Positive and Negative Syndrome Scale (PANSS) was used to evaluate the clinical severity of the disorder. The Clinical Global Impression (CGI) for illness severity and global change was also applied at baseline and week 18 by a neuropsychiatrist. Regular alcohol /tobacco/coffee consumption was assessed by a geneticist through yes/no questions about average weekly consumption during the last 12 months, excluding heavy drinkers and/or smokers.[22] Brief psychotic episodes secondary to intoxication by a psychoactive substance or medical condition were excluded. Other exclusion criteria were significant cognitive deficit (moderate to profound intellectual disability) and brain tumors.

Healthy controls were either college students or unrelated companions of patients recruited at our institution with no family history of neurological or psychiatric disorders, who were not taking psychotropic medications, and who were clinically healthy at the time of sampling.

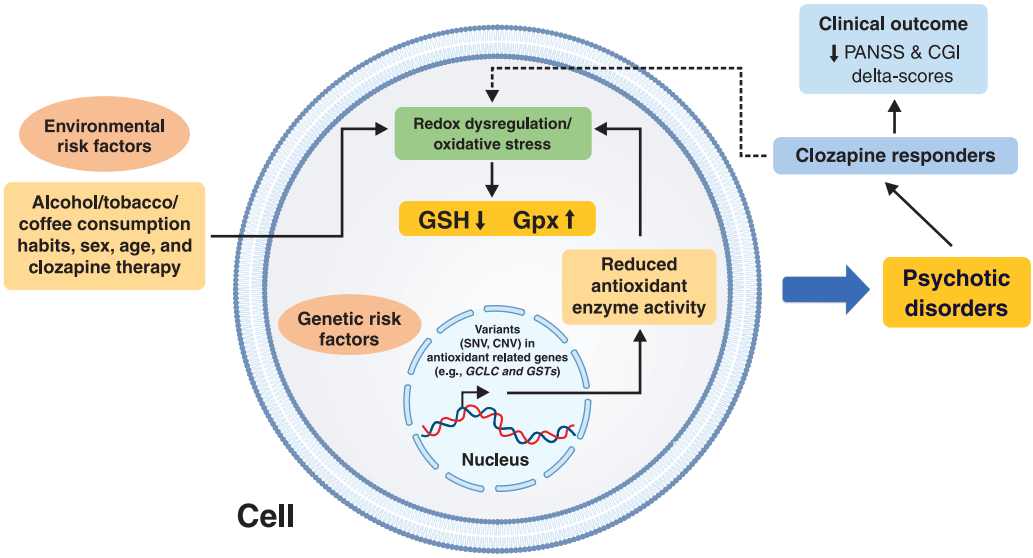

Figure 1 Graphical hypothesis of how genetic and environmental factors converge on the redox system and contribute to the pathophysiology of psychotic disorders. Variants in antioxidant defense-related genes (*GCLC*, *GSTs*, etc.) in combination with environmental factors (alcohol/tobacco/coffee consumption, sex, age, and clozapine therapy) could be implicated via redox-sensitive and oxidative stress-mediated mechanisms, in glutathione (GSH) concentration/glutathione peroxidase (GPx) activity. Potential antioxidant properties of clozapine might modulate the oxidative stress profile and clinical outcome (Positive and Negative Syndrome Scale [PANSS] and Clinical Global Impression Scale [CGI] of responsive patients with psychotic disorders). CNV = copy number variation; SNV = single nucleotide variants.

Biochemical analyses

Blood samples were collected from each participant into ethylenediamine tetraacetic acid (EDTA) or acid-citrate-dextrose tubes for biochemical and molecular assays, respectively. Plasma was separated and stored at -70°C for GSH and GPx analyses.

Measurement of glutathione plasma levels

GSH plasma levels were measured with a previously reported fluorometric method[23,24] using ortho-phthalaldehyde, which was prepared daily in reagent-grade absolute methanol prior to use. Plasma samples were first diluted 1:1 (v/v) with 10% trichloroacetic acid solution and placed on ice for 10 min. The samples were then centrifuged at 4°C and 100,000 *g* for 15 min to obtain the supernatant for the GSH assay. The samples were prepared in a 200 μL 96-well black polystyrene microplate with 13.6 μL of homogenized plasma in 122.7 μL of EDTA-phosphate buffer (pH = 8.0). The ortho-phthalaldehyde solution (13.6 μL) was then added to the mixture and incubated at room temperature for 15 min. Fluorescent signals were recorded in a BioTek™ FLX800 luminescence spectrophotometer at 350 nm excitation and 420 nm emission wavelengths with 90% sensitivity. Calibration curves were built for GSH, and the concentrations were obtained by interpolation of the standard curve. The results were expressed as μmol GSH/L.[23,24]

Plasma glutathione peroxidase activity

Plasma GPx activity was measured using a method based on the nonenzymatic oxidation of GSH.[25] For this purpose, plasma protein content was obtained by the Lowry method using Folin and Ciocalteu's phenol reagent.[26] Samples containing 500 μg of protein were then incubated at 37°C in 250 μL of phosphate buffer (containing 5mM EDTA; pH = 7.0) + 250 μL of GSH (2.0 mM) + 125 μL of NaN_3 (0.01 M) to reach a final volume of 1.5 mL. After 5 min, 250 μL of H_2O_2 (1.25 mM) was added to the incubation medium, and 3 min later, 250 μL of the mixture was removed and diluted in a 250 μL aliquot of buffer. The samples were then centrifuged at 1,500 *g* for 30 min. Supernatants (50 μL) were mixed with 50 μL of phosphate buffer (pH = 7.0). Reaction began after 25 μL of 5,5-dithiobis-2-nitrobenzoic acid was added. After incubation for 2 min at room temperature, optical density was measured at 412 nm in a microplate reader (Eon UV/VIS, Biotek Instruments Inc., Winooski, VT, USA). Calibration curves were built using increasing concentrations of GSH + EDTA (pH = 7.0) + 5,5-dithiobis-2-nitrobenzoic acid, and the concentrations were obtained by interpolation from the standard curve. The results are expressed as μmol of GSH/500 μg protein/30 min.

Determining plasma concentrations of clozapine and N-desmethylclozapine

Plasma concentrations of clozapine and its main metabolite (N-desmethylclozapine) were determined as previously described.[27] We then calculated the metabolic ratio as: metabolic ratio = [clozapine levels]/[N-desmethylclozapine].

Molecular analysis

DNA was isolated by standard procedures. Single nucleotide variants of *GCLC*_rs761142 and rs17883901, and *GSTP1*_rs1695 were genotyped using TaqMan® probes in a StepOne™ real-time polymerase chain reaction (PCR) system (Thermo Fisher Scientific, Waltham, MA, USA). The analysis of CNV of *GSTP1*, *GSTT1*, and *GSTM1* genes was performed by multiplex ligation-dependent probe amplification using a P128 panel (MRC-Holland BV, Amsterdam, The Netherlands) following manufacturer instructions. GAG repeats in 5'UTR of *GCLC* (alleles 7-10 repeats) were analyzed by fluorescent PCR, as previously described.[28] Fluorescent PCR and multiplex ligation-dependent probe amplification products were separated by capillary electrophoresis in an AB3130 genetic analyzer and examined using PeakScaner (Thermo Fisher Scientific, Waltham, MA, USA) and Coffalyser.Net (MRC Holland) software, respectively. A total of 5% of these results were validated by quantitative PCR and direct sequencing, respectively. Precise genotypes of *GSTs*_CNVs were determined using previously published primers for duplex PCR.[29] The characteristics of included genetic variants can be found in Table S1, available as online-only supplementary material.

Statistical analysis

Statistical analysis was performed in R v4.0.2. Categorical variables are presented as numbers and frequencies, and continuous variables as mean values. Descriptive statistics are used for pharmacological and clinical data and alcohol/tobacco/coffee consumption. GSH levels were compared between patients and controls with parametric tests, while GPx activity was assessed with nonparametric tests. Allelic and genotypic frequencies were estimated and compared between groups with the chi-square test; Hardy-Weinberg's equilibrium was tested for all variants in both groups. Differences in GSH levels and GPx activity for each genotype were analyzed in both groups separately; two-way analysis of variance (ANOVA) was also performed to find potential relationships between study group (patients or controls) and genotypes. Absolute levels, adjusted dose-corrected plasma concentrations (ng mL^{-1} per mg kg^{-1}), and the metabolic ratio of clozapine were evaluated along with biochemical parameters for the studied genotypes using analysis of covariance. A comparison of the frequency of individuals carrying each genotype between groups was performed to include significant variants in a subsequent logistic regression analysis. The stepwise logistic regression included the mentioned variants, age, sex, GSH levels, and GPx activity. The best-fitted model was established by comparing the Hosmer-Lemershaw test and Akaike information criteria. Statistical significance was set at $p < 0.05$. To reduce the false discovery rate in

our results, the p-values were corrected for multiple comparisons using the Benjamini-Hochberg procedure.[30]

Ethics statement

This study was conducted in accordance with the declaration of Helsinki and was approved by the Institutional Review Board (National Institute of Neurology and Neurosurgery protocol_104/17). Written informed consent was obtained from all participants prior to enrollment.

Results

The participants' demographic and biochemical data, as well as patients' clinical characteristics and alcohol/tobacco/coffee consumption habits are shown in Table 1.

Table 1 Demographic, biochemical, and clinical characteristics of patients (n=75) and healthy controls (n=40)

Characteristic	Patients mean ± SD (range or %)	Healthy controls mean ± SD (range or %)	p-value (χ^2, *t*, and W values)
Sex (male/female)	47/28 (62.66 males)	25/15 (62.50 males)	1.00‡ (0.00003)
Age (years)	37.11±12.52 (18-73)	33.56±11.86 (18-71)	0.14§ (-1.48)
GSH (μmol/L)	4.29±1.83	5.75±1.44	**0.00001**§ (4.55)
GPx activity†	189.45±98.21	151.82±44.58	**0.03**‖ (915.00)
Weight (kg)	77.96±15.56 (48.50-129.00)		
BMI	28.19±5.44 (16.80-42.20)		
Age at onset (years)	21.88±8.21 (12-49)		
Clinical diagnosis, n (%)			
Schizophrenia	42 (56.00)		
Schizoaffective disorder	15 (20.00)		
Bipolar disorder	7 (9.30)		
Interictal psychosis	11 (14.70)		
Length of illness (years)	16.10±10.07 (2.00-49.00)		
Baseline			
Positive PANSS	32.10±5.45		
Negative PANSS	28.20±6.41		
General PANSS	52.27±5.27		
Total PANSS	113.40±14.42		
CGI-Severity	6.00±0.67		
At week 18			
Positive PANSS	19.86±5.13		
Negative PANSS	12.96±5.81		
General PANSS	40.04±5.65		
Total PANSS	83.18±14.64		
CGI-Change	3.09±1.15		
Patients without additional comedication to clozapine	11 (14.70)		
Patients with additional comedication	64 (85.30)		
Medication, n (%)			
Anxiolytics	35 (46.70)		
Antidepressants	51 (68.00)		
Anticonvulsants	26 (34.70)		
Anticholinergics	2 (2.70)		
Patients who consume, n (%)			
Alcohol	23 (30.70)		
Tobacco	28 (37.30)		
Coffee	49 (65.30)		
Patients with a sedentary lifestyle	50 (66.70)		

Bold type denotes significant values.
BMI = body mass index; CGI = Clinical Global Impression Scale; GSH = plasma glutathione levels expressed as μmol GSH/L; PANSS = Positive and Negative Syndrome Scale.
† Glutathione peroxidase (GPx) activity values are expressed in μmol of GSH/500 μg protein/30 min.
‡ Probability according to Pearson's chi-square test.
§ Probability according to Student's *t*-test.
‖ Probability according to the Mann-Whitney-Wilcoxon test.

The patients' average daily clozapine dose was 184.90± 128.36 mg (range: 10-700 mg) and their mean treatment length was 16.11±10.07 years (range: 2-49 years). The majority of patients were undergoing concomitant pharmacological treatment (85.30%) and showed symptom improvement according to CGI and PANSS scores at week 18 (reductions of 51.50% and nearly 30%, respectively). The patients' average alcohol intake was 1-3 drinks per week and less than 10 cigarettes per day, which is considered light consumption of these substances.[22] All controls and 63% of the patients were nonsmokers.

Patients had lower plasma GSH levels (4.299 ± 1.835 vs. 5.758 ± 1.443 μmol/L; p = 1.49E^{-05}) and higher GPx activity (189.45 ± 98.219 vs. 151.82 ± 44.585 μmol/μg; p = 0.034) than age- and sex-matched healthy controls (Table 1). The mean clozapine levels among patients at steady state were 167.69±192.31 ng mL^{-1} (range = 7.63-865.68 ng mL^{-1}), 184.92±129.36 mg for daily clozapine dose, and 2.10 ± 1.09 (range = 0.33-5.66) for metabolic ratio.

The delta negative PANSS score (Δ = baseline – week 18) was negatively correlated with GPx activity (r = -0.584, p = 0.006) and GSH concentrations (r = -0.500, p = 0.012) (data not shown).

The allele and genotype frequencies of the studied genetic variants were calculated for both groups (Tables S2 and S3). Losses or gains in *GSTP1* were not identified. All variants were in equilibrium according to Hardy-Weinberg's law, except for *GSTM1* gene deletion in the patient group (Table S2). Comparison of allele frequencies between patients and controls only showed a significant difference for the single nucleotide variant *GCLC*_rs761142 (Table 2).

Analysis of GSH levels and GPx activity values by genotype revealed no differences among controls (Table S4), although some differences were observed in the patient group (Table 3). GSH concentrations were higher in carriers of GAG_7/9 repeats in *GCLC* and in carriers of four copies of *GSTM1*. These results remained significant after adjusting for false discovery rate and were confirmed with Tukey test (Table S5). Among patients, differences in GPx activity were observed according to genotypes of *GCLC*_rs761142 variant and CNV of *GSTM1* with the Kruskal-Wallis test (Table 3). The CNV differences in *GSTM1* genotypes were confirmed by false discovery rate correction, showing that homozygous and heterozygous carriers of *GSTM1_del* (0-1 CNV) have similar GPx activity (Table S6). However, the association with the *GCLC*_rs761142 variant did not persist after false discovery rate correction (Table 3 and Table S6). Regarding clinical variables, only the delta values of positive and total PANSS score were associated with *GSTM1*-CNV (p = 0.010 and p = 0.029, respectively) (data not shown).

Two-way ANOVA was performed between groups and genotypes for GSH concentrations and GPx activity. Major differences were observed within and between groups in GSH concentrations, mainly for the genotypes of $(GAG)_n$ repeats in the 5′UTR-*GCLC* (Figures S1 and S2, available as online-only supplementary material). A comparison of the frequencies of the genotypes carried by the individuals in both groups was performed to identify genetic variants and the main differences between them (Table S7). Then, using these results, significant variants were included in a stepwise logarithmic model. The proposed model included GSH concentrations, GPx activity, and sex to get the best-fitted model (Table 4). According to this model, GSH concentration was negatively associated with psychosis (odds ratio [OR] = 0.663, p = 1.00E^{-04}) and the GAG-7/9 *GCLC* genotype (OR = 4.291, p = 0.021), and both heterozygous and homozygous *GCLC*_rs761142 genotypes (OR = 5.888, p = 0.002 and OR = 6.090, p = 0.003, respectively) could be

Table 2 Comparison of allele frequencies of the included genetic variants between patients and controls

		Patients			Healthy controls			
Variant	Allele	n†	Frequency	95%CI	n†	Frequency	95%CI	p-value‡
GCLC gene (GAG)n	7	40	0.340	0.268-0.421	36	0.450	0.346-0.559	0.243 (0.405)
	8	14	0.097	0.058-0.158	8	0.100	0.049-0.187	0.790 (0.850)
	9	81	0.562	0.481-0.641	36	0.450	0.346-0.558	0.122 (0.260)
rs761142	C	95	0.679	0.597-0.750	38	0.475	0.369-0.583	**0.005 (0.050)**
rs17883901	A	8	0.057	0.821-0.929	3	0.037	0.008-0.109	0.723 (0.855)
GSTs gene *GSTP1*_rs1695	G	40	0.435	0.332-0.542	43	0.566	0.447-0.679	0.130 (0.260)
GSTT1 del	del	17	0.181	0.115-0.272	6	0.077	0.032-0.161	0.077 (0.260)
GSTT1 dup	dup	37	0.394	0.301-0.495	28	0.359	0.261-0.470	0.757 (0.855)
GSTM1 del	del	58	0.580	0.482-0.672	46	0.605	0.493-0.708	0.855 (0.855)
GSTM1 dup	dup	6	0.060	0.025-0.127	0	0.000	0.000-0.058	0.079 (0.260)

Bold type denotes significant values.
del = gene deletion; dup = gene duplication; GSTs = glutathione S-transferases.
† Due to DNA quality, the number of patients and controls included in this analysis varied.
‡ Chi-square probability and (adjusted) p-values.

Table 3 Plasma GSH concentrations and GPx activity values stratified by genotype in the group of patients

Variant	Genotype	GSH levels (μmol/L)(mean ± SD)	*t* and F values	p-value†	GPx activity‡ (mean ± SD)	χ^2-value, W-value	p-value§
GCLC gene							
(GAG)n	7/7	3.608±1.086	5.442	**0.0002 (0.0012)**	169.70±29.054	1.889	0.596 (0.688)
	7/8	3.508±1.423			176.00±77.562		
	7/9	**6.038±1.514**‖			226.69±134.508		
	9/9	4.267±1.964			177.30±92.125		
rs761142	AA	4.902±1.544	0.717	0.492 (0.492)	316.90±165.887	7.006	**0.030 (0.090)**
	AC	4.067±1.880			164.72±81.882		
	CC	4.563±1.892			201.50±85.049		
rs17883901	AG	3.823±0.858	-1.2763	0.227 (0.454)	128.44±95.643	81.000	0.057 (0.144)
	GG	4.378±1.934			194.65±91.203		
GSTs gene							
*GSTP1*_rs1695	AA	3.328±0.681	0.79	0.461(0.492)	138.50±53.730	0.749	0.688 (0.688)
	AG	3.191±0.807			153.00±55.420		
	GG	2.929±0.622			146.80±51.260		
*GSTT1*_CNV	0	3.296±0.537	1.232	0.313 (0.469)	185.00±20.518	6.707	0.082 (0.123)
	1	2.160±0.000			NA		
	2	3.132±0.655			127.12±57.905		
	3	3.628±0.674			157.60±36.535		
	4	3.003±0.0897			142.80±53.579		
*GSTM1*_CNV	0	2.994±0.750	6.641	**0.0009 (0.0027)**	153.40±53.024	13.095	**0.004 (0.024)**
	1	3.114±0.814			167.50±46.632		
	2	3.522±0.425			105.60±37.322		
	4	**5.230 ± 0.240**‖			273.80±68.123		

Bold type denotes significant values.
GPx = glutathione peroxidase; GSH = glutathione.
† Analysis of variance (ANOVA) and Student's *t*-tests.
‡ GPx activity is expressed as μmol GSH/500 μg of protein/30 min.
§ Kruskal-Wallis and Mann-Whitney-Wilcoxon probability values and (adjusted) p-values.
‖ Differences vs. all other genotypes.

Table 4 Stepwise logarithmic model

Variable	OR	95%CI	Z-value	p-value†
(intercept)			-1.108	0.267
GSH‡	0.663	0.533-0.807	-3.811	**0.0001**
GPx‡	1.004	1.004-1.014	3.367	**0.0008**
Male sex	0.590	0.290-1.148	-1.554	0.120
*GCLC*_7/8 GAG repeats	1.162	0.423-3.231	0.301	0.763
*GCLC*_7/9 GAG repeats	4.291	1.404-14.319	2.324	**0.020**
*GCLC*_9/9 GAG repeats	1.703	0.729-4.096	1.265	0.206
*GCLC*_rs761142 AC	5.888	1.957-20.923	3.007	**0.003**
*GCLC*_rs761142 CC	6.090	1.931-22.598	2.921	**0.003**

Bold type denotes significant values.
GPx = glutathione peroxidase; GSH = glutathione; OR = odds ratio.
† Probit logarithmic model probability.
‡ Increments of one unit.

additional risk factors for psychosis. Finally, according to the model, GPx activity could also minimally contribute to psychosis (OR = 1.004, p = $8.00E^{-04}$).

Based on these results, the *GCLC*-STR and the *GCLC*-rs761142 genotypes were grouped according to the risk they represent (7/7-7/8 vs. 7/9-9/9, and AA vs. AC-CC) and were then combined to obtain a multilocus genotype. Using these genotype combinations and the assembled groups, the two-way ANOVA was replicated for both GSH levels and GPx activity values (Figure S3). GSH levels showed significant differences between groups for the 7/7.7/8-AC.CC multilocus genotype (p = $1.73E^{-03}$).

The frequency of each multilocus genotype was compared between groups to explore its influence on psychosis risk (Table S8). The *GCLC*_rs761142 variant and the AC-CC multilocus genotype had the highest OR (7/9-9/9 and AC-CC: OR = 10.00, p = 0.003 [adjusted p = 0.0009]).

When the stepwise logarithmic model was replicated using these multilocus genotypes, the significance was lost; only the GSH and GPx values remained associated with psychosis risk (Table S9). GSH levels and GPx activity were compared among patients according to their clinical diagnosis (Figure S4), showing that GSH levels

were different between patients with SD and IIP (ANOVA test p = 0.014; Tukey's test p = 0.007) (Table S10).

Analysis of clozapine levels, adjusted dose-corrected plasma concentrations, and metabolic ratios showed no significant association with the analyzed genetic variants (Table S11).

Discussion

To the best of our knowledge, this is the first report of biochemical and genetic oxidative markers in Mexican patients with refractory psychosis on clozapine monotherapy. We found that *GCLC*_(GAG)n and rs761142 variants were associated with PD risk, which is clinically relevant. A previous Latin-American study on treatment-resistant SZ patients treated with clozapine analyzed CNVs of *GSTT1* and *GSTM1*,[31] and four additional studies assessed GSH levels and certain markers of oxidative stress in treatment-resistant SZ patients.[32-35] Although these studies have similar sample sizes to ours, they only included treatment-resistant patients.

Herein, we confirmed significantly lower plasma GSH levels in patients with PD than in controls, as has been previously reported in peripheral blood, cerebrospinal fluid, and brain tissue.[11,16,18,36-41] However, we also found higher GPx activity in patients than controls. Although it is difficult to make a connection between plasma GSH values and GPx activity and use it to elucidate brain response, it has been proposed that high GPx activity may be a peripheral marker of low brain GSH levels.[16] What is clear is that the antioxidant defense system of PD patients is under oxidative stress. GPx activity in patients with psychosis has shown inconsistent results; some studies and one meta-analysis found no significant differences between GPx levels and other oxidative markers in patients with SZ or early-onset first episode psychosis and controls.[42-44] Three meta-analyses have found lower GPx activity in patients with SZ than controls, which was related to disease chronicity[45] and had a moderate effect size.[46,47] Finally, higher GPx activity has also been found in patients with early onset first psychotic episodes, as well as in patients with SZ and BD, than controls.[12,13,48] Interestingly, one study found that the GPx activity of risperidone-responsive female SZ patients was higher than that of nonresponders, acting as a potential predictor of risperidone response.[49] It is known that antioxidant status is more impaired in drug-free patients than medicated patients, indicating that antipsychotics may differentially improve antioxidant defense systems.[47] Our patients were taking clozapine as antipsychotic monotherapy. It has been shown that clozapine protects neuron-like rat pheochromocytoma cells from death due to oxidative stress induced by H_2O_2 via a cell-type specific mechanism involving inhibition of extracellular signal-regulated kinase phosphorylation.[50] In addition, a comparative study of the effects of six atypical antipsychotics, including clozapine, on markers of oxidative stress in patients with SZ suggested beneficial antioxidative action by reducing lipid peroxidation and increasing total plasma antioxidant activity.[35] Moreover, postmortem brain studies of patients with SZ or BD using microarray technologies have identified *GCLM* as a differentially expressed gene, upregulated in clozapine-treated patients compared to those treated with other atypical antipsychotics. *GCLM* encodes the glutamate-cysteine ligase modifier, a key enzyme in GSH synthesis.[51] It has also been reported that clozapine upregulates GPx-1 and Nrf2-dependent synthetic GSH systems in mice.[52] Collectively, these studies suggest that *GCLM* upregulation could be induced by clozapine, causing the observed increase in GPx activity, although this must be proven. Of note, GPx activity and GSH levels were higher in patients with lower negative PANSS delta values. Alternatively, increased GPx antioxidant activity may reflect prior cellular oxidative stress or serve as a compensatory mechanism in patients with PD.[12] One study included a subgroup of patients with temporal lobe epilepsy and IIP, who were characterized by increased oxidative stress toxicity and lower antioxidant defenses.[5] In the present study, GSH levels were higher in patients with IIP than those with SD (p = 0.014) (Table S9), suggesting that the oxidative pathophysiology of IIP differs from the oxidative stress profile observed in SD. Further studies with larger samples should explore this.

Our study hypothesis, that some variants of genes involved in GSH metabolism may influence the risk of psychosis and oxidative stress profile of clozapine-treated patients, was confirmed for two *GCLC* variants in this sample of Mexican patients with PD (Figure 1).

The intronic single nucleotide variant *GCLC*_rs761142 was more frequent in patients than controls (p = 0.005) (Table 2). This variant has been associated with lower *GCLC* mRNA expression, deficient elimination of neurotoxicants,[53] and sulfamethoxazole-induced hypersensitivity in patients with human immunodeficiency virus/acquired immune deficiency syndrome.[54] *GCLC* encodes the glutamate-cysteine ligase catalytic subunit, a rate-limiting enzyme involved in GSH biosynthesis. The GAG repeat variant in the 5' untranslated region of *GCLC* has also been linked to decreased GSH production[28] and has been associated with SZ risk.[16,18] Thus, individuals with high-risk *GCLC* genotypes (7/8, 8/8, 8/9, and 9/9 GAG repeats) have shown lower GSH levels in the pregenual anterior cingulate cortex regardless of illness condition.[18] In the present study, GSH concentrations were only higher in patients with the low-risk *GCLC* genotype (GAG-7/9 repeats) or carriers of four copies of *GSTM1* (Table 3 and Table S4), which agrees with previous reports.[55,56] An association between the *GCLC* genotype GAG-7/9 and higher GSH levels was not observed in the healthy control group. All of our patients were responsive to clozapine, and a potential link between clozapine response and *GCLC* genotype has been proposed.[33] However, there was no significant association between genetic variants and clozapine levels, clozapine adjusted dose-corrected plasma concentrations, or metabolic ratios (Table S11). Future studies in larger samples should confirm our results.

On the other hand, some studies have reported that the nonfunctional or null alleles of *GST*s, as well as the single nucleotide variant of *GSTP1*-rs1695; p.Ile105Val, which affects its enzyme activity, are related to higher

susceptibility to treatment-resistant SZ.[31,57,58] GST enzymes play relevant roles in protecting cells against oxidative stress, catalyzing the conjugation of reduced GSH in a wide variety of substrates.[59] In line with this, differences in GPx activity were detected after comparing the *GCLC*_rs761142 and *GSTM1*_CNV genotypes (Table 4). Regarding CNV in GSTs, only *GSTM1*_CNV was positively correlated with GSH levels and GPx activity: the higher the number of copies, the higher the values of both factors. In addition, higher *GSTM1*-CNV expression was associated with better response to clozapine, according to PANSS delta values. The *GSTM1* results are also controversial. For instance, combined analysis of CNV in *GSTM1* and *GSTT* has shown a significant association between nonnull genotypes at both *loci* and an additive effect for increased vulnerability to SZ in Spanish patients.[60] However, the same combinations of null alleles were associated in Tunisian and Iranian patients with BD,[61,62] and the *GSTM1*-null genotype has been independently associated with the risk of early-onset mental disorders in Serbian patients.[17] Subsequently, a case-control study and a meta-analysis failed to find a significant association between the null genotype of *GSTM1* and SZ in a Japanese sample.[63] These discrepancies may be due to ethnic differences in genotypic distribution of *GST*s-CNVs.[64]

The contradictory results for oxidative stress markers may be explained by sample sizes, ethnicity, methodological differences, and the inclusion of confounding variables. In this context, some limitations of our study include its small sample size, the fact that several confounding variables were not controlled for, the lack of a group of nonresponders or patients treated with a different antipsychotic, and that genetic variants and activities of other antioxidant enzymes were excluded (e.g., superoxide dismutase, nitric oxide synthase, or catalase).

Of note, one study reported that patients with SZ who were taking clozapine had higher plasma GSH levels than those taking risperidone.[65] We did not include a patient group who took different antipsychotics to confirm this finding. Nevertheless, we believe that our results are important for future studies and are clinically relevant.

Overall, the genetic variants of *GCLC* analyzed in this study were associated with the oxidative stress profile of patients with refractory psychosis, which confirms our study hypothesis. Clinicians should be aware of GSH concentrations/GPx activity and *GCLC* genotype to classify patients with PD, allowing opportunities for interventions that could improve their antioxidant defenses.

Disclosure

The authors report no conflicts of interest.

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