



# Accessing Gene Expression in Treatment-Resistant Schizophrenia

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## Abstract

Schizophrenia (SCZ) is a mental disorder arising from a complex interaction of genetic and environmental factors. It has been suggested that treatment-resistant schizophrenia (TRS) is a distinct, more severe, and homogenous subgroup of schizophrenia that could present specific biological markers. Our aim was to characterize expression of target genes in blood of TRS patients compared with non-TRS (NTRS) patients and healthy controls (HC). TRS has been defined using failure to respond to two previous antipsychotic trials. We hypothesized that genes involved in neurodevelopment, myelination, neuroplasticity, neurotransmission, and miRNA processing could be involved in treatment resistance; then, we investigated 13 genes related to those processes in 256 subjects, being 94 healthy controls and 162 schizophrenia patients treated with antipsychotics. Of those, 78 were TRS patients and 84 were NTRS patients. Peripheral blood samples were collected from all subjects and RNA was isolated. Gene expression analysis was performed using the *TaqMan low-density array* (TLDA) technology. To verify the influence of expression quantitative trait loci (eQTLs), we evaluated single-nucleotide polymorphism (SNP) of all genes using data from GTEx Project. SNP genotypes were obtained from HumanOmniExpress BeadChip. We did not detect gene expression differences between TRS and NTRS subjects, indicating candidate genes specific to treatment resistance. We detected an upregulation of *CNR1* and *UFDIL* gene expression in patients (TRS and NTRS groups) when compared to controls, that may be associated with the release of neurotransmitters, which can influence neuronal plasticity, or with a stress response-activating protein degradation. *DICER1* and *AKT1* expression increased slightly across the groups and could differentiate only the extreme opposite groups, HC and TRS. Both genes act in heterogeneous pathways, such as cell signaling and miRNA processing, and seem to have an increased demand in the TRS group. We did not detect any eQTLs in our sample that could explain differences in mRNA levels, suggesting a possible regulation by other mechanism, not driven by genotypes. Our data strengthen the importance of several biological pathways involved in the schizophrenia refractoriness and severity, adding knowledge to develop more effective treatments in the future.

**Keywords** Gene expression · Treatment-resistant schizophrenia · eQTLs

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## Introduction

Schizophrenia (SCZ) is a complex disease with a heterogeneous psychopathology. Several hypotheses have been raised about the etiology of SCZ. In neurodevelopment hypothesis, it is proposed that the disease has its origins in disturbed development of the nervous system prior to the onset of clinical symptoms, due to a combination of genetic and environmental factors [1].

Although genetic factors are considered the strongest risk factor for SCZ [2], finding the genetic etiology of the disease is still a challenge due to the complex combination of many common genetic variants of small effect (predominantly single-nucleotide polymorphisms—SNPs), rare variants with high predictive value (such as copy number variations—CNVs), and epigenetics [3]. Furthermore, complex diseases such as SCZ tend to result not only from alterations in individual genes, but from changes in molecular networks [4].

Antipsychotic drugs in current use can be classified into two categories: typical antipsychotics and atypical antipsychotics. The two classes have similar efficacy in the control of positive and negative symptoms, but differ in the propensity to trigger extrapyramidal symptoms [5]. Antipsychotic therapy is an important approach in SCZ treatment, but psychotic relapse rates remain high. It is estimated that 20–30% of patients with schizophrenia do not respond to treatment with conventional antipsychotics [6, 7].

The treatment-resistant schizophrenia (TRS) is defined by an inadequate response to a sequence of treatments [8]. An inadequate response needs not to be restricted to the persistence of positive symptoms, but this is the most common definition. The concept of treatment resistance should not be confused with “chronic schizophrenia” as chronicity and “resistance” are different concepts. Chronic patients can respond to standard treatments and treatment resistance may be as high as 15% even in first-episode patients [9]. Studies have shown that individuals with a family history of psychosis are more likely to poorly respond to antipsychotic drugs [10, 11], suggesting a genetic influence in the development of TRS. As TRS seem to be a distinct and homogeneous subgroup of schizophrenia [12, 13], studies have focused on finding biological markers that could predict the response to antipsychotics and consequently improve the prognosis of the patients. Our main aim was to compare expression of target genes in blood among TRS patients, non-TRS (NTRS) patients, and healthy controls (HC) and to find eQTLs that could have an influence on the expression of those genes.

## Materials and Methods

### Study Population

SCZ group was comprised of chronically outpatients recruited from Schizophrenia Program (PROESQ) of UNIFESP, treated

with diverse types of typical and atypical antipsychotics. The diagnosis of SCZ was established according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) using the Structured Clinical Interview (SCID-I). Treatment-resistant (TR) status was defined using modified criteria of The International Psychopharmacology Algorithm Project (IPAP)—schizophrenia algorithm. Patients were regarded as TRS in the presence of (a) no response to at least two antipsychotics used in monotherapy for a minimum period of 4 to 6 weeks with appropriate doses of the drug and (b) presence of moderate to severe psychopathology, especially positive symptoms [14]. Antipsychotic types, doses, and number of medications were registered and compared between the TRS and NTRS groups. To evaluate the effect of dosages of different medications in mRNA expression, chlorpromazine (CPZ) equivalent doses were estimated using regression equations according to Andreasen et al. (2010) [15] and were compared between the groups. In patients using more than one type of medication, CPZ doses were calculated, summing equivalent doses of each antipsychotic. Patients were also evaluated using standardized instruments, including the Positive and Negative Syndrome Scale (PANSS) and Global Assessment of Functioning (GAF). The HC group comprised volunteers with no current or previous psychiatric diagnoses or first-degree family history of psychotic disorders. The Research Ethics Committee of UNIFESP approved the research protocol, and all participants and family members provided written informed consent prior to enrolment in the study (CAAE 06191612.7.0000.5505).

### Genetic Analyses

A total of 5 mL of whole blood was collected in PAXgene® RNA tubes (PreAnalytix, Hombrechtikon, Switzerland) and RNA was isolated using the PAXgene® Blood RNA kit (Qiagen, Germantown, MD), according to the manufacturer’s instructions. RNA quality and integrity was evaluated using electrophoresis on a 1.0%-agarose gel and NanoDrop® ND-1000 spectrophotometer (NanoDrop, Wilmington, DE), respectively.

Approximately 400 ng of each RNA sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA). Then, 20 to 100 ng of cDNA was diluted in H<sub>2</sub>O, mixed with the TaqMan® Universal PCR Master Mix (Life Technologies, Foster City, EUA), and loaded on TaqMan low-density array (TLDA) microfluidic cards (Life Technologies, Foster City, EUA). Probes and primers of 13 target genes, 2 housekeeping genes (HKGs: *ACTB* and *GAPDH*), and 1 positive control for the reaction (*18S*) were preloaded in the 384 wells of each TLDA card (Life Technologies). The HKGs were selected based on the detection of a strong correlation of relative threshold method (C<sub>rt</sub>) values between *ACTB* and *GAPDH*

genes in blood, no association of both gene Crt values with SCZ diagnosis and psychopathology symptoms, and the detection of smallest stability values using NormFinder [16]. We selected the candidate genes based on a review of the literature considering the following aspects: (i) at least one report on association with SCZ, (ii) plausibility of their involvement in SCZ pathophysiology, and (iii) expression in blood according to information in the Anatomy tool of GENEVESTIGATOR [17]. Genes that presented medium to high mRNA levels in blood in this tool were defined as expressed in whole blood. We selected genes related to neurotransmission (*COMT* and *CNR1*), inflammation and the immune system (*TNF*), neurodevelopment (*DISC1*, *PAFAH1B1*, and *NDEL1*), myelination (*MBP*), cell signaling (*AKT1*), the microRNA machinery (*DGCR8*, *DICER1*, and *DROSHA*), protein degradation (*UFDIL*), and adhesion (*DGCR2*). Some genes were selected mainly because of being located in the genomic region known as the most important genetic risk factor for psychotic disorders: the 22q11.2 region (*COMT*, *DGCR2*, *DGCR8*, and *UFDIL*) [18]. The experiments were performed in accordance with the manufacturer's instructions using the ViiA™ 7 Real-Time PCR System (Life Technologies, Foster City, EUA).

Gene expression was quantified using the Crt, discounting the *ACTB* and *GAPDH* geometric mean (GM) as endogenous controls ( $\Delta\text{Crt} = \text{Crt}_{\text{target gene}} - \text{Crt}_{\text{GM}}$ ). To each sample, a  $\Delta\text{Crt}$  was calculated and a fold change (FC) was calculated using  $2^{-\Delta\Delta\text{Crt}}$ , always considering the HC group as reference. Gene expression and  $\Delta\text{Crt}$  values are negatively correlated; therefore, higher  $\Delta\text{Crt}$  values mean lower gene expression.

Polymorphisms located within those 13 target genes were extracted from the Genotyping Array using PLINK software version 2.0, and the correlations between their genotype and tissue-specific gene expression levels described in this manuscript were obtained from the Single-Tissue eQTLs and/or Gene eQTL Visualizer on GTEx Portal on dbGaP Accession phs000424.v6.p1.

## Statistical Analysis

The statistical analyses were performed in the Statistical Package for the Social Sciences (SPSS), version 15.0 dataset.

**Table 1** Descriptive analysis of sex and age of the HC, NTRS, and TRS groups

	HC		NTRS		TRS		$X^2$	$p^a$
	<i>N</i>	%	<i>N</i>	%	<i>N</i>	%		
Sex								
M	53	56.40	52	61.90	50	64.10	1.16	0.560
F	41	43.60	32	38.10	28	35.90		
	<i>N</i>	Mean (SD)	<i>N</i>	Mean (SD)	<i>N</i>	Mean (SD)	$F_{(WG;BW)}$	$p^b$
Age (y)	94	35.9 (11.4)	84	39.35 (11.2)	78	38.18 (10.5)	2.278 (253;2)	0.105

*F* female, *M* male, *HC* healthy controls, *TRS* treatment-resistant schizophrenia, *NTRS* non-treatment-resistant schizophrenia, *SD* standard deviation, *y* years,  $X^2$  chi-squared value,  $p^a$  *p* value obtained in chi-squared test,  $F$  *F* value obtained in ANOVA comparison among groups,  $p^b$  *p* value obtained in ANOVA test,  $w_G$  degree of freedom within groups,  $w_B$  degree of freedom between groups

Gender differences between the SCZ and HC groups were verified using the chi-squared test. Age, disease, and treatment duration were compared among the three groups (TRS, NTRS, and HC) using analysis of variance (ANOVA) test and between two groups (TRS and NTRS) using Student's *t* test.  $\Delta\text{Crt}$  values were compared among each group using the ANOVA, and post hoc comparisons were carried out using the Bonferroni test. Pearson correlation was used to test correlations between mRNA levels and PANSS scores, GAF scores, and CPZ equivalent doses.

## Results

### Samples

We recruited a total of 94 healthy controls and 162 SCZ patients, 78 of whom were TRS patients and the other 84 were NTRS patients. The characteristics of samples from SCZ patients (TRS and NTRS) and controls are described in Table 1. Sex (chi-squared = 1.160;  $p = 0.560$ ) and age ( $F_{(253,2)} = 2.278$ ;  $p = 0.105$ ) did not differ among the groups.

The number of antipsychotics being used between the TRS and NTRS groups were different, with a smaller number of TRS subjects being treated in monotherapy ( $N = 49$ ; 43.4%) in comparison to that of NTRS subjects ( $N = 64$ ; 56.6%), and a higher number of TRS patients ( $N = 26$ ; 70.3%) being treated with two or more antipsychotics than that of NTRS patients ( $N = 11$ ; 29.7%) (chi-squared = 9.288;  $p = 0.002$ ). In monotherapy, olanzapine and risperidone accounts for the treatment of 50 and 18.8% of the 64 NTRS subjects, respectively. In the TRS group, clozapine was the antipsychotic used by 77.6%, followed by olanzapine, used by 8.2% of the 49 patients. We observed differences in CPZ equivalent doses of antipsychotics between the groups, with TRS patients receiving higher doses of medication ( $t = 6.421$ ;  $df 142$ ;  $p < 0.001$ ) than NTRS patients (Table 2).

No differences in disease or treatment duration between the TRS and NTRS groups were detected. We observed that TRS patients are affected earlier than NTRS patients, with mean age of onset smaller than NTRS patients ( $t 2.142$ ;  $df 160$ ;  $p =$

**Table 2** Comparison of clinical variables between patients in the NTRS and TRS groups

	NTRS		TRS		$t_{(df)}$	$p$
	$N$	Mean (SD)	$N$	Mean (SD)		
Age of onset	84	23.0 (11.5)	78	19.4 (10.1)	2.142 <sub>(160)</sub>	0.034
SCZ duration (y)	82	14.3 (9.4)	73	16.3 (7.8)	-1.430 <sub>(153)</sub>	0.155
Treatment duration (y)	68	13.1 (9.0)	63	14.6 (7.1)	-1.102 <sub>(129)</sub>	0.272
PANSS	80	58.8 (19.0)	75	71.6 (19.8)	-4.116 <sub>(153)</sub>	<0.001
GAF	57	56.3 (15.5)	52	45.0 (14.4)	3.924 <sub>(107)</sub>	<0.001
CPZ equivalent dose	68	375.5 (261.5)	76	699.7 (334.8)	-6.421 <sub>(142)</sub>	<0.001

SCZ schizophrenia, CPZ chlorpromazine, TRS treatment-resistant schizophrenia, NTRS non-treatment-resistant schizophrenia, SD standard deviation, y years, PANSS Positive and Negative Syndrome Scale, GAF Global Assessment of Functioning,  $df$  degree of freedom,  $t$   $t$  value obtained in Student's  $t$  test,  $p$   $p$  value obtained in Student's  $t$  test

0.034). As expected, total PANSS ( $t = 4.116$ ;  $df = 153$ ;  $p < 0.001$ ) and GAF ( $t = 3.924$ ;  $df = 107$ ;  $p < 0.001$ ) scores were higher in TRS patients in comparison to NTRS patients (Table 2).

### Gene Expression and eQTL Analyses

Regarding treatment resistance, we did not observe gene expression differences between the TRS and NTRS subjects. We also did not detect gene expression differences between patients in use of clozapine compared to other antipsychotic drugs. Although differences between CPZ equivalent doses were detected between the groups, no correlation was observed between CPZ equivalent doses and mRNA levels of the 13 genes analyzed ( $p > 0.05$ ).

We detected differences in *CNR1* ( $F_{(174,2)} = 7.00$ ;  $p = 0.001$ ) and *UFDIL* ( $F_{(237,2)} = 12.69$ ;  $p < 0.001$ ) gene expression among the HC, TRS, and NTRS groups. The expression of *CNR1* and *UFDIL* increases proportionately with the severity of the disease, being upregulated in patients (NTRS and TRS) in relation to HC (Table 3). However, there was no correlation between *CNR1* and *UFDIL* mRNA levels and psychopathology, measured by PANSS or GAF ( $p > 0.05$ ). Fold change and ANOVA  $p$  values, after Bonferroni post hoc test, of *CNR1* (NTRS: FC = 1.91,  $p = 0.004$ ; and TRS: FC = 1.96,  $p = 0.002$ ) and *UFDIL* (NTRS: FC = 1.18,  $p = 0.01$ ; and TRS: FC = 1.31,  $p < 0.001$ ) are presented in Fig. 1(a, b).

We observed differences in mRNA levels of *AKT1* ( $F_{(240,2)} = 4.47$ ;  $p = 0.01$ ) and *DICER1* ( $F_{(241,2)} = 3.81$ ;  $p = 0.02$ ) between the HC and TRS group. Both genes were upregulated in the TRS group in comparison to the HC group. Fold change and ANOVA  $p$  values, after Bonferroni post hoc test, of *AKT1* (FC = 1.11,  $p = 0.01$ ) and *DICER1* (FC = 1.13,  $p = 0.03$ ) are presented in Fig. 1(c, d).

According to the GTEx database, the 13 genes studied present 25 eQTLs of brain and blood tissues. Of those, we had genotype information from 19 SNPs; however, none of

them were situated in genes differently expressed among the groups.

### Discussion

Although TRS patients present characteristics that support a categorically different illness subtype [19], such as gray matter alterations [20], glutamatergic abnormalities [21], and a lack of dopaminergic abnormalities [22, 23], in our study, we did not observe differences in whole blood gene expression that could discriminate TRS and NTRS patients in a very specific manner. However, we found two genes probably associated with disease pathophysiology, *CNR1* and *UFDIL*, that were differentially expressed in the blood of HC subjects, when compared to TRS and NTRS patients, and other two genes, *AKT1* and *DICER1*, that were differentially expressed between the extreme groups HC and TRS. *CNR1* gene is located on 6q14–15, a region of replicated linkage for SCZ, and encodes type-1 cannabinoid receptors (CB1) that are activated by endocannabinoids neurotransmitters, by cannabinoids such as the compound delta-9-tetrahydrocannabinol (THC) and by synthetic analogues of THC [24].

*CNR1* is proposed as a candidate gene to SCZ etiology since the endocannabinoid system has emerged as a possible link between cannabis and psychosis, especially after the comprehension that the psychogenic properties of cannabis are mainly mediated by THC stimulation of CB1 [25]. Some SNPs of this gene have been associated with central nervous system effects [24, 26, 27] with SCZ subtypes [28–30] and treatment refractoriness [31]. However, it is not clear how the genetic variants could influence in disease development, since most of them are not related to amino acid substitution and protein structure changes [24]. Stress induced by maternal diet deprivation seems to trigger executive function deficits associated to an overexpression of *Cnr1*, in mouse prefrontal cortex (PFC) [32]. These executive function deficits, although unspecific, are associated with different psychiatric diagnoses

**Table 3** Gene expression comparison among the HC, TRS, and NTRS groups using ANOVA test

Genes	HC			NTRS			TRS			ANOVA statistical analysis		
	<i>N</i>	Mean $\Delta$ Crt	SD	<i>N</i>	Mean $\Delta$ Crt	SD	<i>N</i>	Mean $\Delta$ Crt	SD	<i>F</i>	<i>df</i> ( <sub>WG;BG</sub> )	<i>p</i>
<i>COMT</i>	94	5.56	0.26	77	5.41	0.86	75	5.40	0.44	2.05	(243; 2)	0.13
<i>TNF</i>	91	6.88	0.37	72	6.83	0.42	74	6.71	0.66	2.45	(234; 2)	0.09
<i>CNR1</i>	43	11.81	0.82	67	10.88	1.90	67	10.84	1.23	7.00	(174; 2)	0.001*
<i>AKT1</i>	93	4.38	0.24	75	4.28	0.29	75	4.22	0.47	4.47	(240; 2)	0.012*
<i>MBP</i>	79	8.67	0.62	64	8.73	0.52	68	8.73	0.65	0.21	(208; 2)	0.81
<i>DGCR8</i>	92	6.94	0.40	74	7.05	0.42	71	7.02	0.66	1.16	(234; 2)	0.32
<i>DICER1</i>	94	5.46	0.35	75	5.34	0.39	75	5.29	0.54	3.81	(241; 2)	0.023*
<i>DROSHA</i>	90	6.49	0.41	73	6.52	0.39	74	6.48	0.63	0.12	(234; 2)	0.89
<i>DGCR2</i>	94	4.72	0.39	75	4.83	0.48	75	4.83	0.55	1.41	(241; 2)	0.25
<i>UFD1L</i>	92	7.16	0.45	74	6.92	0.42	74	6.77	0.64	12.69	(237; 2)	<0.001*
<i>DISC1</i>	83	8.50	0.81	73	8.08	1.51	71	8.10	0.99	3.50	(224; 2)	0.03
<i>NDEL1</i>	94	4.10	0.28	75	4.03	0.39	75	3.97	0.47	2.34	(241; 2)	0.10
<i>PAFAH1B1</i>	94	4.65	0.22	76	4.44	1.14	75	4.55	0.49	1.89	(242; 2)	0.15

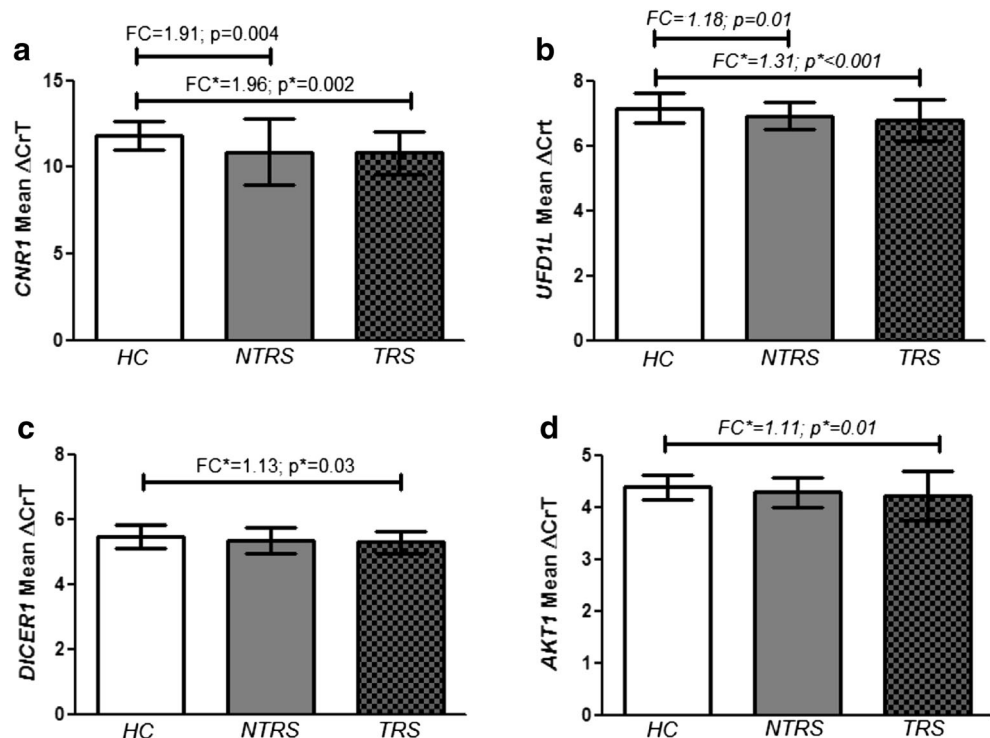
*N* number of subjects in each group, *SD* standard deviation,  $\Delta$ Crt delta relative threshold obtained in real-time PCR, *HC* healthy controls, *TRS* treatment-resistant schizophrenia, *NTRS* non-treatment-resistant schizophrenia, *F* *F* value obtained in ANOVA comparison among groups, *p* *p* value obtained in ANOVA comparison among groups, *df* degree of freedom, *WG* within groups, *BG* between groups

\*Significant *p* values after Bonferroni post hoc test

[33–35]. Increased expression of *Cnr1* gene was also reported in the frontal cortex of male rats after maternal diet deprivation, associated with depressive-like and psychotic-like symptoms [36]. Upregulation of *CNR1* was also described in peripheral blood mononuclear cells (PBMCs) of SCZ patients in comparison to HC, but not in subjects with bipolar disorder

types I and II, or major depressive disorder (MDD) compared to controls [37]. Supporting this upregulation, decreased promoter DNA methylation was observed in *CNR1* of those SCZ patients. Similarly, to what is observed in humans, increased expression of *Cnr1* was detected in the PFC of rats exposed to methylazoxymethanol acetate (MAM), a

**Fig. 1** Comparison of *CNR1* (a), *UFD1L* (b), *DICER1* (c), and *UFD1L* (d) mRNA levels among groups.  $\Delta$ Crt and gene expression are inverse correlated; therefore, TRS group exhibits the highest expression levels of all genes.  $\Delta$ Crt delta relative threshold obtained in real-time PCR, *HC* healthy controls, *TRS* treatment-resistant schizophrenia, *NTRS* non-treatment-resistant schizophrenia, *p* significance level for ANOVA test after post hoc comparison between NTRS and HC groups, *p*\* significance level for ANOVA test after post hoc comparison between TRS and HC groups, *FC* mRNA expression fold change of NTRS group using HC as reference, *FC*\* mRNA expression fold change of TRS group using HC as reference



developmental animal model of SCZ, compared to a control group of animals. Less DNA methylation at promoter region was also reported in *Cnr1* gene of this animal model of SCZ. It is important to highlight that these changes in mRNA expression and DNA methylation were investigated in other genes which are part of endocannabinoid system, such as endocannabinoid receptor 2 (CB2) among others, but only *CNR1* was significantly changed [37]. CB1 receptors regulate the release of inhibitory and excitatory neurotransmitters such as glutamate, dopamine, and gamma-aminobutyric acid (GABA) that can influence neuronal plasticity [38] and may be important to SCZ development. *UFDIL* gene is located at chromosome 22q11.2, whose deletion is responsible for the DiGeorge syndrome, considered one of the main genetic risk factors for SCZ [39]; hence, genes located in this region may contribute to SCZ susceptibility [40]. *UFDIL* gene encodes for the human homolog of yeast ubiquitin-fusion-degradation 1 protein, expressed from fetal to post-natal period [41], possibly acting in neurodevelopment. It is suggested that *UFDIL* is directly associated with stress response in endoplasmic reticulum (ER)-associated degradation (ERAD) [42], since this protein, in complex with others, extracts misfolded proteins from ER to be degraded in cytosol by the proteasome [43]. Polymorphism of *UFDIL* was previously associated with the disease [40, 44], with age of onset [40], and cognitive deficits in SCZ [45], but not to treatment resistance. In other study of our group, increased *UFDIL* expression was described in early stage of psychosis, comparing gene expression between individuals in ultra-high risk (UHR), those with first episode of psychosis (FEP), and HC. Higher levels of *UFDIL* mRNA was observed in those in UHR when compared to other groups, and pointed this gene as candidate as biomarker of prodromal phases of SCZ. Similarly to the findings in the UHR study, the upregulation of *UFDIL* observed in our patients could be associated with an increased demand of *UFDIL* protein in response to cellular stress, via ERAD pathways. Although reinforcing this gene importance in SCZ etiology, it is not clear how *UFDIL* mRNA expression varies across SCZ stages.

Beyond genes possibly related to the disease, we also detected alterations in mRNA levels of *DICER1* and *AKT1* genes in the blood of HC when compared to TRS. *DICER1* and *AKT1* expressions increased slightly across groups, and could differentiate only the extreme opposite groups, HC and TRS, not being associated with treatment resistance itself. Both genes were upregulated in first episode of psychosis patients who met bipolar disorder (with psychotic symptoms) diagnostic criteria in comparison to first episode of psychosis patients with SCZ diagnosis and HC [46]. Since *AKT1* and *DICER1* act in heterogeneous processes, it is not clear if the absence of differences between TRS and NTRS was due to

some variable not evaluated in the TRS group, such as presence of other comorbidities and use of other pharmacological drugs, among others.

*DICER1* is a ribonuclease that is central in the biogenesis of miRNAs, being responsible for the processing of the pre-miRNA hairpin structure to form mature miRNAs. Upregulation of *DICER1* was already shown in dorsolateral prefrontal cortex [47, 48] and lymphoblastoid cell lines of SCZ patients [49]. Upregulation of several specific miRNAs was described both in the brain and blood of SCZ subjects [50], and in plasma of TRS individuals compared to NTRS individuals [51]. The upregulation of *DICER1* levels observed in our TRS patients could be associated with an increased demand of enzymes that process miRNAs throughout the disease evolution, being more pronounced in the TRS group.

*AKT1* is a member of the serine-threonine protein kinase involved in cell signaling and neural development [52]. Increased activity of AKT genes has been associated with several human diseases, including different types of cancer [53, 54]. Impairment of AKT signaling pathway was described in a number of brain disorders, including SCZ [54, 55], in which decreased protein and mRNA levels of *AKT1* were reported in the blood and brain [56, 57]. Activation of *AKT1* was described in the brain of mice after antipsychotic treatment [58], reaffirming a role of *AKT1* in SCZ development. In the present study, different than others [58–60], we detected an increase of *AKT1* mRNA levels in SCZ patients, specifically TRS patients, compared only to HC.

We did not detect any eQTLs in our sample that might explain differences in mRNA levels, suggesting a possible regulation by other mechanism, not driven by genotypes. Following this line, D'Addario and co-workers presented evidences of epigenetic regulation of *CNR1* expression in humans and animal model [37]. Other agents, like virus and vitamins, can also change the expression of *DICER1* [61, 62].

Our sample of TRS patients was affected earlier by SCZ in comparison to NTRS patients, and presented more severe psychopathology, in line to other cases described in literature, and reinforcing a possible pronounced genetic basis of the TR outcome [63–65].

In the present study, we detected differences of *CNR1* and *UFDIL* expressions in the blood of healthy subjects and SCZ patients, independently of treatment response, highlighting the importance of these genes to SCZ etiological mechanisms. Besides, we also detected differences of *AKT1* and *DICER1* mRNA expressions between TRS patients and HC, showing these genes might be related to the severity rather than to the disease itself. All genes were upregulated in TRS compared to HC and seem to present a slightly increased expression throughout the disease progression.

Although these findings should be interpreted in light of some limitations, like the heterogeneity of disease and treatment resistance, and the absence of data related to possible

confounders (like the use of medications not related to antipsychotic, BMI index, and the presence of other comorbidities), our results can provide clues of biological pathways involved in TRS that could contribute to better understand treatment response and to develop more effective treatments in the future.

**Compliance with Ethical Standards** The Research Ethics Committee of UNIFESP approved the study, and all the participants and family members provided written informed consent prior to enrolment in the study (CAAE 06191612.7.0000.5505).

## References

- Owen MJ, O'Donovan MC, Thapar A, Craddock N (2011) Neurodevelopmental hypothesis of schizophrenia. *Br J Psychiatry* 198(3):173–175. <https://doi.org/10.1192/bjp.bp.110.084384>
- Henriksen MG, Nordgaard J, Jansson LB (2017) Genetics of schizophrenia: overview of methods, findings and limitations. *Front Hum Neurosci* 11:322. <https://doi.org/10.3389/fnhum.2017.00322>
- Gejman PV, Sanders AR, Kendler KS (2011) Genetics of schizophrenia: new findings and challenges. *Annu Rev Genomics Hum Genet* 12(1):121–144. <https://doi.org/10.1146/annurev-genom-082410-101459>
- Boyle EA, Li YI, Pritchard JK (2017) An expanded view of complex traits: from polygenic to omnigenic. *Cell* 169(7):1177–1186. <https://doi.org/10.1016/j.cell.2017.05.038>
- Leucht S, Pitschel-Walz G, Abraham D, Kissling W (1999) Efficacy and extrapyramidal side-effects of the new antipsychotics olanzapine, quetiapine, risperidone, and sertindole compared to conventional antipsychotics and placebo. A meta-analysis of randomized controlled trials. *Schizophr Res* 35(1):51–68. [https://doi.org/10.1016/S0920-9964\(98\)00105-4](https://doi.org/10.1016/S0920-9964(98)00105-4)
- Kapur S, Seeman P (2001) Does fast dissociation from the dopamine d(2) receptor explain the action of atypical antipsychotics?: a new hypothesis. *Am J Psychiatry* 158(3):360–369. <https://doi.org/10.1176/appi.ajp.158.3.360>
- Kane JM, Honigfeld G, Singer J, Meltzer H (1988) Clozapine in treatment-resistant schizophrenics. *Psychopharmacol Bull* 24(1):62–67
- Taylor DM, Duncan-McConnell D (2000) Refractory schizophrenia and atypical antipsychotics. *J Psychopharmacol* 14(4):409–418. <https://doi.org/10.1177/026988110001400411>
- McGorry P, Killackey E, Elkins K, Lambert M, Lambert T (2003) Summary Australian and New Zealand clinical practice guideline for the treatment of schizophrenia. *Australasian Psychiatry* 2(11):136–147
- Crespo-Facorro B, de la Foz VO, Ayesa-Arriola R, Perez-Iglesias R, Mata I, Suarez-Pinilla P et al (2013) Prediction of acute clinical response following a first episode of non affective psychosis: results of a cohort of 375 patients from the Spanish PAFIP study. *Prog Neuro-Psychopharmacol Biol Psychiatry* 44:162–167. <https://doi.org/10.1016/j.pnpbp.2013.02.009>
- Huber CG, Naber D, Lambert M (2008) Incomplete remission and treatment resistance in first-episode psychosis: definition, prevalence and predictors. *Expert Opin Pharmacother* 9(12):2027–2038. <https://doi.org/10.1517/14656566.9.12.2027>
- Meltzer HY (1999) The role of serotonin in antipsychotic drug action. *Neuropsychopharmacology* 21(2 Suppl):106S–115S. [https://doi.org/10.1016/S0893-133X\(99\)00046-9](https://doi.org/10.1016/S0893-133X(99)00046-9)
- Meltzer HY (1990) Defining treatment refractoriness in schizophrenia. *Schizophr Bull* 16(4):563–565. <https://doi.org/10.1093/schbul/16.4.563>
- IPAP. The International Psychopharmacology Algorithm Project. 2009 [cited 2017 21/12/2017]; Available from: <http://www.ipap.org/schiz/index.php>.
- Andreasen NC, Pressler M, Nopoulos P, Miller D, Ho BC (2010 Feb 1) Antipsychotic dose equivalents and dose-years: a standardized method for comparing exposure to different drugs. *Biol Psychiatry* 67(3):255–262. <https://doi.org/10.1016/j.biopsych.2009.08.040>
- Spindola LM, Pan PM, Moretti PN, Ota VK, Santoro ML, Cogomora H, Gadelha A, Salum G et al (2017) Gene expression in blood of children and adolescents: mediation between childhood maltreatment and major depressive disorder. *J Psychiatr Res* 92:24–30. <https://doi.org/10.1016/j.jpsychires.2017.03.015>
- Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L et al (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv Bioinforma* 2008:420747
- Christofolini DM, Bellucco FT, Ota VK, Belangero SI, Cernach MC, Gadelha A et al (2011) Assessment of 22q11.2 copy number variations in a sample of Brazilian schizophrenia patients. *Schizophr Res* 132(1):99–100. <https://doi.org/10.1016/j.schres.2011.07.007>
- Gillespie AL, Samanaite R, Mill J, Egerton A, MacCabe JH (2017) Is treatment-resistant schizophrenia categorically distinct from treatment-responsive schizophrenia? A systematic review. *BMC Psychiatry* 17(1):12. <https://doi.org/10.1186/s12888-016-1177-y>
- Anderson VM, Goldstein ME, Kydd RR, Russell BR (2015) Extensive gray matter volume reduction in treatment-resistant schizophrenia. *Int J Neuropsychopharmacol* 18(7):pyv016
- Demjaha A, Egerton A, Murray RM, Kapur S, Howes OD, Stone JM, McGuire PK (2014) Antipsychotic treatment resistance in schizophrenia associated with elevated glutamate levels but normal dopamine function. *Biol Psychiatry* 75(5):e11–e13. <https://doi.org/10.1016/j.biopsych.2013.06.011>
- Demjaha A, Murray RM, McGuire PK, Kapur S, Howes OD (2012) Dopamine synthesis capacity in patients with treatment-resistant schizophrenia. *Am J Psychiatry* 169(11):1203–1210. <https://doi.org/10.1176/appi.ajp.2012.12010144>
- Krebs MO, Sautel F, Bourdel MC, Sokoloff P, Schwartz JC, Olie JP et al (1998) Dopamine D3 receptor gene variants and substance abuse in schizophrenia. *Mol Psychiatry* 3(4):337–341. <https://doi.org/10.1038/sj.mp.4000411>
- Gouvea ES, Santos AFF, Ota VK, Mrad V, Gadelha A, Bressan RA et al (2017) The role of the CNR1 gene in schizophrenia: a systematic review including unpublished data. *Rev Bras Psiquiatr* 39(2):160–171. <https://doi.org/10.1590/1516-4446-2016-1969>
- D'Souza DC, Abi-Saab WM, Madonick S, Forselius-Bielen K, Doersch A, Braley G et al (2005) Delta-9-tetrahydrocannabinol effects in schizophrenia: implications for cognition, psychosis, and addiction. *Biol Psychiatry* 57(6):594–608. <https://doi.org/10.1016/j.biopsych.2004.12.006>
- Dinu IR, Popa S, Bicu M, Mota E, Mota M (2009) The implication of endocannabinoid system effects. *Rom J Intern Med* 47(1):9–18
- Okahisa Y, Kodama M, Takaki M, Inada T, Uchimura N, Yamada M, Iwata N, Iyo M et al (2011 Mar) Association study of two cannabinoid receptor genes, CNR1 and CNR2, with methamphetamine dependence. *Curr Neuropharmacol* 9(1):183–189. <https://doi.org/10.2174/157015911795017191>
- Ujike H, Takaki M, Nakata K, Tanaka Y, Takeda T, Kodama M, Fujiwara Y, Sakai A et al (2002) CNR1, central cannabinoid receptor gene, associated with susceptibility to hebephrenic schizophrenia. *Mol Psychiatry* 7(5):515–518. <https://doi.org/10.1038/sj.mp.4001029>

29. Chavarria-Siles I, Contreras-Rojas J, Hare E, Walss-Bass C, Quezada P, Dassori A et al (2008) Cannabinoid receptor 1 gene (CNR1) and susceptibility to a quantitative phenotype for hebephrenic schizophrenia. *Am J Med Genet B Neuropsychiatr Genet* 147(3):279–284. <https://doi.org/10.1002/ajmg.b.30592>
30. Martinez-Gras I, Hoenicka J, Ponce G, Rodriguez-Jimenez R, Jimenez-Arriero MA, Perez-Hernandez E et al (2006) (AAT)n repeat in the cannabinoid receptor gene, CNR1: association with schizophrenia in a Spanish population. *Eur Arch Psychiatry Clin Neurosci* 256(7):437–441. <https://doi.org/10.1007/s00406-006-0665-3>
31. Hamdani N, Tabeze JP, Ramoz N, Ades J, Hamon M, Sarfati Y, Boni C, Gorwood P (2008) The CNR1 gene as a pharmacogenetic factor for antipsychotics rather than a susceptibility gene for schizophrenia. *Eur Neuropsychopharmacol* 18(1):34–40. <https://doi.org/10.1016/j.euroneuro.2007.05.005>
32. Grissom NM, Herdt CT, Desilets J, Lidsky-Everson J, Reyes TM (2015) Dissociable deficits of executive function caused by gestational adversity are linked to specific transcriptional changes in the prefrontal cortex. *Neuropsychopharmacology* 40(6):1353–1363. <https://doi.org/10.1038/npp.2014.313>
33. Kuntsi J, Frazier-Wood AC, Banaschewski T, Gill M, Miranda A, Oades RD, Roeyers H, Rothenberger A et al (2013) Genetic analysis of reaction time variability: room for improvement? *Psychol Med* 43(6):1323–1333. <https://doi.org/10.1017/S0033291712002061>
34. Johnson KA, Robertson IH, Kelly SP, Silk TJ, Barry E, Daibhis A et al (2007) Dissociation in performance of children with ADHD and high-functioning autism on a task of sustained attention. *Neuropsychologia* 45(10):2234–2245. <https://doi.org/10.1016/j.neuropsychologia.2007.02.019>
35. Ben Shalom D, Ronel Z, Faran Y, Meiri G, Gabis L, Kerns KA (2017) A double dissociation between inattentive and impulsive traits, on tasks of visual processing and emotion regulation. *J Atten Disord* 21(7):543–553. <https://doi.org/10.1177/1087054713510351>
36. Marco EM, Echeverry-Alzate V, Lopez-Moreno JA, Gine E, Penasco S, Viveros MP (2014) Consequences of early life stress on the expression of endocannabinoid-related genes in the rat brain. *Behav Pharmacol* 25(5–6):547–556. <https://doi.org/10.1097/FBP.000000000000068>
37. D'Addario C, Micale V, Di Bartolomeo M, Stark T, Pucci M, Sulcova A, Palazzo M, Babinska Z et al (2017) A preliminary study of endocannabinoid system regulation in psychosis: distinct alterations of CNR1 promoter DNA methylation in patients with schizophrenia. *Schizophr Res* 188:132–140. <https://doi.org/10.1016/j.schres.2017.01.022>
38. Wilson RI, Nicoll RA (2002) Endocannabinoid signaling in the brain. *Science* 296(5568):678–682. <https://doi.org/10.1126/science.1063545>
39. Bassett AS, Chow EW, Husted J, Weksberg R, Caluseriu O, Webb GD, Gatzoulis MA (2005) Clinical features of 78 adults with 22q11 deletion syndrome. *Am J Med Genet A* 138(4):307–313. <https://doi.org/10.1002/ajmg.a.30984>
40. Ota VK, Belangero SI, Gadelha A, Bellucco FT, Christofolini DM, Mancini TI, Ribeiro-dos-Santos AK, Santos SE et al (2010) The UFD1L rs5992403 polymorphism is associated with age at onset of schizophrenia. *J Psychiatr Res* 44(15):1113–1115. <https://doi.org/10.1016/j.jpsychires.2010.04.008>
41. Novelli G, Mari A, Amati F, Colosimo A, Sanguiuolo F, Bengala M, Conti E, Ratti A et al (1998) Structure and expression of the human ubiquitin fusion-degradation gene (UFD1L). *Biochim Biophys Acta* 1396(2):158–162. [https://doi.org/10.1016/S0167-4781\(97\)00211-X](https://doi.org/10.1016/S0167-4781(97)00211-X)
42. Chen M, Gutierrez GJ, Ronai ZA (2011) Ubiquitin-recognition protein Ufd1 couples the endoplasmic reticulum (ER) stress response to cell cycle control. *Proc Natl Acad Sci U S A* 108(22):9119–9124. <https://doi.org/10.1073/pnas.1100028108>
43. Ye Y, Meyer HH, Rapoport TA (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature*. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.] 414(6864):652–656
44. De Luca A, Pasini A, Amati F, Botta A, Spalletta G, Alimenti S et al (2001) Association study of a promoter polymorphism of UFD1L gene with schizophrenia. *Am J medical Genetics*. [Multicenter Study Research Support, Non-U.S. Gov't] 105(6):529–533
45. Ota VK, Berberian AA, Gadelha A, Santoro ML, Ottoni GL, Matsuzaka CT, Mari JJ, Melaragno MI et al (2013 Aug 30) Polymorphisms in schizophrenia candidate gene UFD1L may contribute to cognitive deficits. *Psychiatry Res* 209(1):110–113. <https://doi.org/10.1016/j.psychres.2013.03.035>
46. Gouvea ES, Ota VK, Noto C, Santoro ML, Spindola LM, Moretti PN, Carvalho CM, Xavier G et al (2016 Oct 04) Gene expression alterations related to mania and psychosis in peripheral blood of patients with a first episode of psychosis. *Transl Psychiatry* 6(10):e908. <https://doi.org/10.1038/tp.2016.159>
47. Santarelli DM, Beveridge NJ, Tooney PA, Cairns MJ (2011) Upregulation of dicer and microRNA expression in the dorsolateral prefrontal cortex Brodmann area 46 in schizophrenia. *Biol Psychiatry* 69(2):180–187. <https://doi.org/10.1016/j.biopsych.2010.09.030>
48. Beveridge NJ, Gardiner E, Carroll AP, Tooney PA, Cairns MJ (2010) Schizophrenia is associated with an increase in cortical microRNA biogenesis. *Mol Psychiatry* 15(12):1176–1189. <https://doi.org/10.1038/mp.2009.84>
49. Sanders AR, Goring HH, Duan J, Drigalenko EI, Moy W, Freda J et al (2013) Transcriptome study of differential expression in schizophrenia. *Hum Mol Genet* 22(24):5001–5014. <https://doi.org/10.1093/hmg/ddt350>
50. Camkurt MA, Karababa F, Erdal ME, Bayazit H, Kandemir SB, Ay ME et al (2016) Investigation of dysregulation of several microRNAs in peripheral blood of schizophrenia patients. *Clin Psychopharmacol Neurosci* 14(3):256–260. <https://doi.org/10.9758/cpn.2016.14.3.256>
51. Alacam H, Akgun S, Akca H, Ozturk O, Kabukcu BB, Herken H (2016) miR-181b-5p, miR-195-5p and miR-301a-3p are related with treatment resistance in schizophrenia. *Psychiatry Res* 245:200–206. <https://doi.org/10.1016/j.psychres.2016.08.037>
52. Balu DT, Carlson GC, Talbot K, Kazi H, Hill-Smith TE, Easton RM, Birnbaum MJ, Lucki I (2012 Feb) Akt1 deficiency in schizophrenia and impairment of hippocampal plasticity and function. *Hippocampus* 22(2):230–240. <https://doi.org/10.1002/hipo.20887>
53. Woodgett JR (2005) Recent advances in the protein kinase B signaling pathway. *Curr Opin Cell Biol* 17(2):150–157. <https://doi.org/10.1016/j.ceb.2005.02.010>
54. Arguello PA, Gogos JA (2008) A signaling pathway AKTing up in schizophrenia. *J Clin Invest* 118(6):2018–2021. <https://doi.org/10.1172/JCI35931>
55. Emamian ES (2012) AKT/GSK3 signaling pathway and schizophrenia. *Front Mol Neurosci* 5:33
56. Aubry JM, Schwald M, Ballmann E, Karege F (2009) Early effects of mood stabilizers on the Akt/GSK-3beta signaling pathway and on cell survival and proliferation. *Psychopharmacology* 205(3):419–429. <https://doi.org/10.1007/s00213-009-1551-2>
57. Tan HY, Nicodemus KK, Chen Q, Li Z, Brooke JK, Honea R, Kolachana BS, Straub RE et al (2008) Genetic variation in AKT1 is linked to dopamine-associated prefrontal cortical structure and function in humans. *J Clin Invest* 118(6):2200–2208. <https://doi.org/10.1172/JCI34725>
58. Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat Genet* 36(2):131–137. <https://doi.org/10.1038/ng1296>



59. Karege F, Perroud N, Schurhoff F, Meary A, Marillier G, Burkhardt S et al (2010) Association of AKT1 gene variants and protein expression in both schizophrenia and bipolar disorder. *Genes Brain Behav* 9(5):503–511. <https://doi.org/10.1111/j.1601-183X.2010.00578.x>
60. van Beveren NJ, Buitendijk GH, Swagemakers S, Krab LC, Roder C, de Haan L et al (2012) Marked reduction of AKT1 expression and deregulation of AKT1-associated pathways in peripheral blood mononuclear cells of schizophrenia patients. *PLoS One* 7(2): e32618. <https://doi.org/10.1371/journal.pone.0032618>
61. Casseb SM, Simith DB, Melo KF, Mendonca MH, Santos AC, Carvalho VL, Cruz ACR, Vasconcelos PFC (2016) Drosha, DGCR8, and Dicer mRNAs are down-regulated in human cells infected with dengue virus 4, and play a role in viral pathogenesis. *Genet Mol Res* 15(2). <https://doi.org/10.4238/gmr.15027891>
62. Dambal S, Giangreco AA, Acosta AM, Fairchild A, Richards Z, Deaton R, Wagner D, Vieth R et al (2017) microRNAs and DICER1 are regulated by 1,25-dihydroxyvitamin D in prostate stroma. *J Steroid Biochem Mol Biol* 167:192–202. <https://doi.org/10.1016/j.jsbmb.2017.01.004>
63. Meltzer HY, Rabinowitz J, Lee MA, Cola PA, Ranjan R, Findling RL, Thompson PA (1997 Apr) Age at onset and gender of schizophrenic patients in relation to neuroleptic resistance. *Am J Psychiatry* 154(4):475–482. <https://doi.org/10.1176/ajp.154.4.475>
64. Lieberman JA, Safferman AZ, Pollack S, Szymanski S, Johns C, Howard A, Kronig M, Bookstein P et al (1994) Clinical effects of clozapine in chronic schizophrenia: response to treatment and predictors of outcome. *Am J Psychiatry* 151(12):1744–1752. <https://doi.org/10.1176/ajp.151.12.1744>
65. Quintero J, Barbudo del Cura E, Lopez-Ibor MI, Lopez-Ibor JJ (2011) The evolving concept of treatment-resistant schizophrenia. *Actas Esp Psiquiatr* 39(4):236–250