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Gene expression imputation provides clinical and biological insights into treatment-resistant schizophrenia polygenic risk

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ABSTRACT

Genome-wide association studies (GWAS) have revealed the polygenic nature of treatment-resistant schizophrenia TRS. Gene expression imputation allowed the translation of GWAS results into regulatory mechanisms and the construction of gene expression (GReX) risk scores (GReX-RS). In the present study we computed GReX-RS from the largest GWAS of TRS to assess its association with clinical features. We perform transcriptome imputation in the largest GWAS of TRS to find GReX associated with TRS using brain tissues. Then, for each tissue, we constructed a GReX-RS of the identified genes in a sample of 254 genotyped first episode of psychosis (FEP) patients to test its association with clinical phenotypes, including clinical symptomatology, global functioning and cognitive performance. Our analysis provides evidence that the polygenic basis of TRS includes genetic variants that modulate the expression of certain genes in certain brain areas (substantia nigra, hippocampus, amygdala and frontal cortex), which at the same time are related to clinical features in FEP patients, mainly persistence of negative symptoms and cognitive alterations in sustained attention, which have also been suggested as clinical predictors of TRS. Our results provide a clinical explanation of the polygenic architecture of TRS and give more insight into the biological mechanisms underlying TRS.

1. Introduction

The cornerstone treatment for psychotic disorders is antipsychotic drugs. Unfortunately, around 20–30 % of patients do not respond to antipsychotic medication and are considered to have treatment-resistant schizophrenia (TRS) (Siskind et al., 2022). TRS is commonly defined as a less than 20 % reduction of positive symptoms after at least two trials of non-clozapine antipsychotics, each at an adequate dose and duration (Nucifora et al., 2019). Some clinical confounders complicate the identification of TRS (i.e. treatment non-adherence, inadequate dosage or duration of treatment, potential comorbid factors) (Potkin et al., 2020). TRS patients have poorer outcomes (i.e. persistent positive, negative and cognitive symptoms, worse social functioning and long-term disability) and 3–11-fold more annual medical and socio-economic costs than schizophrenia patients in remission(Kennedy et al., 2014).

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Clinical evidence suggests two distinct patterns of TRS; most individuals seem to have TRS from the onset of illness, that is, patients that not respond to antipsychotic medications from the start of first treatment (also considered early or primary treatment resistant). Other clinical samples have shown to present a late or secondary treatment resistance, as they present a transition to treatment resistance having initially responded to antipsychotic medications (Ajnakina et al., 2020; Correll and Howes, 2021; Lally et al., 2016; Potkin et al., 2020). As the gold standard, clozapine is the first drug prescribed for TRS, being the only evidence-based antipsychotic for this condition. Around 30–60 % of TRS patients respond to this second-generation antipsychotic(Gillespie et al., 2017). However, clozapine prescription is one of the areas in schizophrenia with the greatest mismatch between its well-demonstrated efficacy and underutilization in clinical practice (Rubio and Kane, 2020). Several studies have reported a mean delay in clozapine initiation of 2–4 years; longer delays in clozapine treatment contribute to poorer treatment outcomes (Shah et al., 2018). The underuse and long delay in clozapine initiation have been attributed to the side effects of clozapine, mainly agranulocytosis or severe neutropenia that requires blood monitoring, and clinician-related factors, such as lack of experience with prescribing clozapine (Farooq et al., 2019; Verdoux et al., 2018). In contrast, antipsychotic polypharmacy is commonly prescribed in treatment-resistant schizophrenia, despite the lack of sufficient evidence for its efficacy (Howes et al., 2017).

It is essential to elucidate the underlying pathophysiology of TRS and to identify its predictors and biomarkers for its early detection and treatment (Potkin et al., 2020). In this context, the identification of factors associated with TRS are timely and praiseworthy research challenges, while first episode samples represent a unique opportunity for estimating the individual risk for early treatment-resistant schizophrenia (Lally et al., 2016).

Several markers of TRS have been identified, mainly clinical and demographic markers, such as a longer duration of untreated psychosis (DUP), more severe negative symptomatology and cognitive alterations, younger age at illness onset, and poorer premorbid functioning (Yang et al., 2022). Some biological features of TRS have also been identified, including lower dopamine synthesis in the associative striatum, higher glutamate neurometabolite levels in the anterior cingulate cortex and reduced gray matter volume (Wada et al., 2022).

Regarding genetic studies, no candidate gene has been robustly associated with TRS (Gillespie et al., 2017). Recently, several authors have explored the hypothesis that TRS is a more severe form of schizophrenia with a strong element of genetic susceptibility (Facal and Costas, 2023). These studies have shown a modest association of the schizophrenia polygenic risk score (SZ-PRS) with TRS (Facal and Costas, 2023; Lin et al., 2023). As an alternative to the SZ-PRS, a TRS-PRS was constructed in the largest genetic study of TRS performed to date (Pardiñas et al., 2022). This PRS was calculated from an interaction test, calculating differences in effect sizes of two case-control genome-wide association studies (GWAS) where the case samples were defined as individuals with TRS and individuals without TRS. Although no locus was significantly associated with TRS, the constructed PRS was associated with TRS in two independent samples (Pardiñas et al., 2022).

Although GWAS and the PRS provide evidence of the complex genetic architecture of TRS, they cannot explore gene- or tissue-level associations. In contrast, transcriptomic imputation allows the identification of tissue-specific gene associations (Wainberg et al., 2019). Transcriptome imputation uses gene expression predictor models derived from large datasets (e.g. Gene-Tissue Expression project (GTEx)) to predict genetically regulated gene expression (GReX) of specific tissues in genotyped cohorts without collecting tissue samples (Huckins et al., 2019; Johnson et al., 2022). This allow the advancement of Transcriptome-Wide Association Study (TWAS) in these cohorts. TWAS can provide a more comprehensive understanding of the genetic basis of complex traits and diseases by considering gene expression patterns in addition to genetic variations. In addition, using a similar approach to that used for the construction of the PRS, an imputed gene expression risk score (GReX-RS) can be computed for specific tissues (Gusev et al., 2018; Huckins et al., 2019; Pain et al., 2021; Rodriguez-López et al., 2020). These scores, based on large numbers of genes, explained more variance in schizophrenia risk than those based solely on genes significantly associated with the disease (Rodriguez-López et al., 2020).

The aim of the present study is to investigate the biological and clinical aspects of TRS within a polygenic framework. The primary hypothesis is that genetic variations associated with TRS will impact the gene expression in specific brain regions, which in turn may influence clinical features that extend beyond the complex phenotype of TRS. These clinical features are present since the first episode of psychosis. To address this hypothesis, the study employed a multi-step approach; (1) The study began by conducting transcriptome imputation. This involves using genetic data from the largest genome-wide association study GWAS of TRS (Pardiñas et al., 2022) to identify GReX associated with TRS. (2) In each of the identified brain tissues from step 1, a GReX-RS was constructed using the genes associated with TRS. This step aimed to quantify the collective gene expression pattern of these genes in the brain tissues. (3) The association between the GReX-RS constructed and various clinical phenotypes, including clinical symptomatology, global functioning and cognitive performance, was tested in a sample of patients with first episode psychosis (FEP) (Mas et al., 2020) (Fig. 1).

2. Material and methods

2.1. Association analysis of GREX with TRS

We performed transcriptome imputation using S-PrediXcan (Barbeira et al., 2018) in the summary statistics from the largest GWAS of TRS (Pardiñas et al., 2022). Then, we performed a TWAS testing for the association of GReX with TRS using prediction models (MASHR-based models) trained on nine brain tissues based on GTEx v8 release data (Aguet et al., 2020) downloaded from the PredictDB repository: Putamen (PUT), Substantia Nigra (SN), Prefrontal Cortex (FL), Hypothalamus (HTH), Hippocampus (HIP), Cerebellum (CB), Caudate (CAU), Anterior Cingulate Cortex (CNG) and Amygdala (AMY). The analysis was performed following the standard procedure, as described by the authors (https://github.com/hakyimlab/S-PrediXcan). The number of predicted GReX varied among tissues: PUT 2053, SN 1243, FL 2314, HTH 1720, HIP 1751, CB 4188, CAU 2707, CNG 2054 and AMY 1442. We applied the genome wide significant threshold of p-value$= 5\times10^{-8}$.

2.2. Samples

A total of 335 FEP patients participated in the 'Phenotype-Genotype Interaction: Application of a Predictive Model in First Psychotic Episodes' (PEPs based on Spanish acronym) (Bernardo et al., 2019, 2013), a collaborative project between various members of the Spanish Research Network on Mental Health (CIBERSAM) (Salagre et al., 2019). This was a multicentre, naturalistic, prospective and longitudinal study.

The following inclusion criteria were used: 1) age between 7 and 35 years at the first evaluation stage; 2) <12 months' history of psychotic symptoms; 3) fluent in Spanish and 4) provision of written informed consent (in the case of children under 16 years of age, parents or legal guardians gave written informed consent). Exclusion criteria were as follows: 1) intellectual disability according to DSM-IV-TR criteria; 2) history of head trauma with loss of consciousness and 3) organic disease with mental repercussions.

In the present study, 254 Caucasian patients (age>16) who provided blood samples for genetic analysis and passed the genetic quality control (see section on blood sampling and genotyping) were included.

The PEPs Project was approved by the Clinical Research Ethics Committee of all participating clinical centers and was conducted in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice.

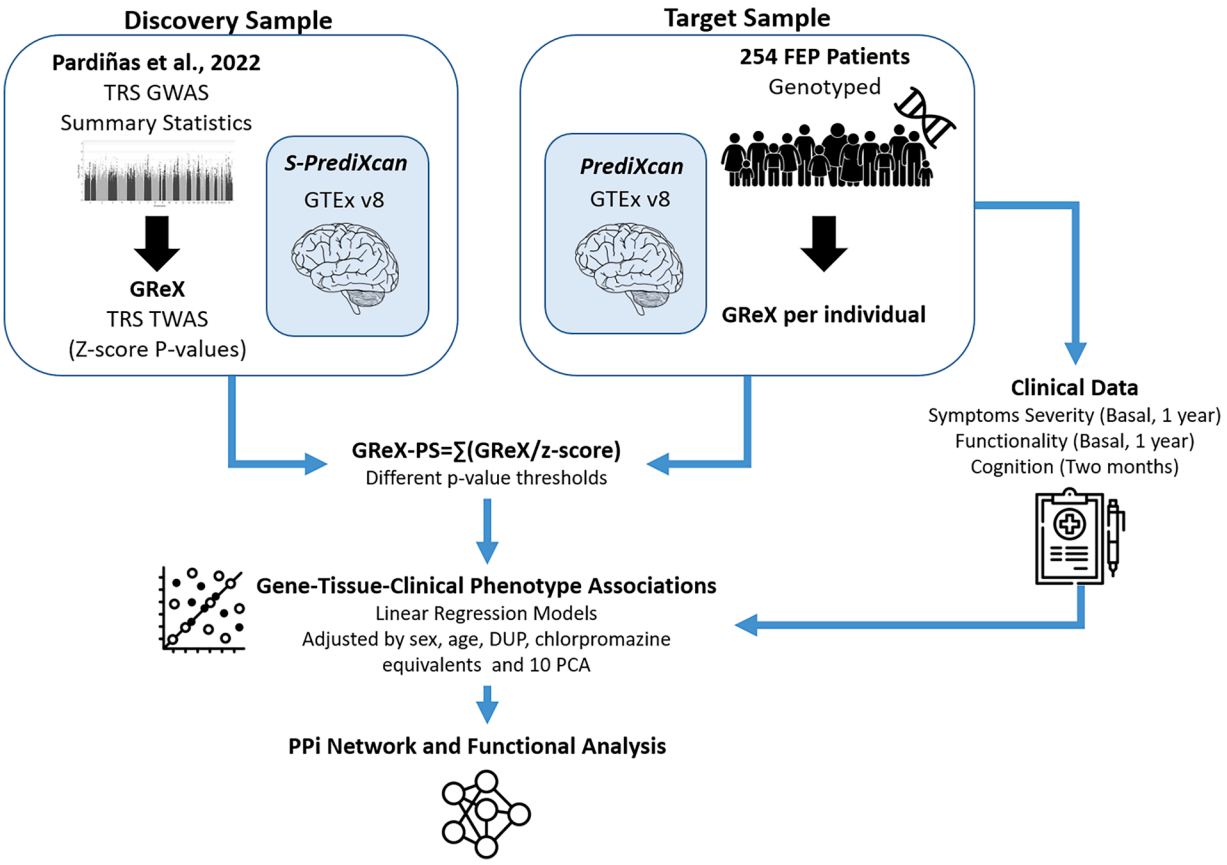


Fig. 1. Flowchart of the study. GReXs associated with TRS in the discovery sample based on the GWAS summary statistics were imputed using S-PrediXcan and GTEx brain models (Pardiñas et al., 2022). In the target sample (Mas et al., 2020), GReX per individual was estimated and the GReX-RS was calculated. We tested the association of GReX-RSs with clinical phenotypes including psychotic symptomatology, global functioning and cognition. Finally, to explore the biological properties of the genes included in significant GReX-RSs, PPi networks were constructed. DUP, duration of untreated psychosis; FEP, first episode of psychosis; GReX, genetically regulated gene expression; GTEX, Gene-Tissue Expression project; GWAS, genome-wide association study; TRS, treatment-resistant schizophrenia; PCA, principal component analysis; PPi, protein–protein interaction; TWAS, transcriptome-wide association study.

Pharmacological treatment was measured using chlorpromazine equivalents (CPZ) based on international consensus (Gardner et al., 2010). To calculate the DUP, the number of days between the first appearance of psychotic symptoms and the date when starting treatment for psychosis was considered.

2.2.1. *Psychopathology assessments*

Diagnoses were established using the Structured Clinical Interview for DSM (SCID-I-II) (First et al., 1997) according to DSM-IV criteria. The Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987) was administered for the psychopathology assessment at baseline and follow-up after 2 and 6 months, and after 1 and 2 years. For the present study, due to the potential loss of sample at 2 years, we use symptomatology data for a period of 1 year.

Although the PANSS is one of the most widely used measures of negative symptom severity, it has been demonstrated that it has several limitations; for instance, it was not designed to evaluate negative symptoms exclusively (Marder and Kirkpatrick, 2014). Thus, we also used the PANSS-Marder Factor Score (Marder et al., 1997) as it has more restrictive criteria to assess positive and negative symptomatology. The sum of the following items of the PANSS was used to calculate the Positive Symptom Factor (PSF): delusions (P1), hallucinatory behavior (P3), grandiosity (P5), suspiciousness/persecution (P6), stereotyped thinking (N7), somatic concerns (G1), unusual thought content (G9) and lack of judgment and insight (G12); and for the Negative Symptom Factor (NSF): blunted affect (N1), emotional withdrawal (N2), poor rapport (N3), passive/apathetic social withdrawal (N4), lack of spontaneity and conversation flow (N6), motor retardation (G7) and active social avoidance (G16).

2.2.2. *Functional assessment*

The Functioning Assessment Short Test (FAST) (Rosa et al., 2007) was administered to evaluate overall functioning at baseline and 1-year follow-up. The FAST contains 24 items, rated from 0 (no difficulty) to 3 (severe difficulty), and assesses impairment or disability across the following six areas of functioning: autonomy (make decisions, speak and act on one's own behalf, without interference from outside sources), occupational functioning (maintain a paid job; efficiency of work performance; working in one's chosen field of study; earning in line with level of employment); cognitive functioning (ability to concentrate, perform simple mental calculations, solve problems, learn new information, and remember learned information), management of personal finances (the capacity to manage finances and spend in a balanced and controlled fashion); interpersonal relationships (relationships with partner, friends, family, involvement in social activities, sexual relations, and the ability to defend ideas and opinions), and leisure time (engaging in physical activities such as sport and exercise, and enjoying hobbies). Higher scores indicate poorer functioning.

2.2.3. *Cognitive assessment*

Cognitive assessment was performed 2 months after the baseline visit in order to ensure the clinical stability of patients. The neuropsychological battery measured the following cognitive domains: (1) sustained attention, assessed with the Continuous Performance Test–II (CPT-II)

(Conners et al., 2003), version 5; (2) verbal learning ability and episodic memory, evaluated with the Verbal Learning Test Spain Complutense for adults (TAVEC); (3) working memory, based on the Digit Span Subtest and the Letter-Number Sequencing Subtest of the Wechsler Adult Intelligence Scale (WAIS-III) (Wechsler, 1997); and (4) executive functioning, evaluated using the Wisconsin Card Sorting Test (Heaton, 1993), corrected by age and educational level. Higher scores corresponded to better performance in all cognitive domains except for attention.

A factorial analysis was performed between the neuropsychological battery tests identifying the four cognitive domains: attention, verbal learning and memory, working memory and executive function. Additionally, to summarize the information about the principal cognitive domains, a global cognitive composite score was calculated as the arithmetic mean of the four domains. All tests and measures used for domain summary scores have been described previously (Bernardo et al., 2013; Cuesta et al., 2015).

2.2.4. Blood samples, genotyping and PRS construction

K2EDTA BD Vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, New Jersey) were used to collect blood samples, which were subsequently stored at −20 °C prior to shipment to the central laboratory for further analysis. The MagNA Pure LC DNA isolation kit – large volume and MagNA Pure LC 2.0 Instrument (Roche Diagnostics GmbH, Mannheim, Germany) were used for DNA extraction, and DNA concentration was determined by absorbance using NanoDrop ND1000 (NanoDrop, Wilmington, Delaware). Specifically, 2.5 μg of genomic DNA was sent to the Spanish National Genotyping Centre (CeGen) for genotyping using the Axiom™ Spain Biobank Array (developed in the University of Santiago de Compostela, Spain).

Genotyping data were submitted to the Michigan Imputation Server (Das et al., 2016), following the standard pipeline for Minimac4 software and setting a European population reference from build GRCh37/hg19, reference panel HRC 1.1 2016 and Eagle v2.4 phasing. Quality control was performed with PLINK v1.07 (Purcell et al., 2007). Inclusion criteria for SNPs were minor allele frequency (MAF) > 0.01, Hardy-Weinberg equilibrium $p > 10^{-6}$, marker missingness $\langle 0.01$ and imputation INFO $\rangle 0.8$. Pruning was done using a window/step size of 200/50 kb and r2 > 0.25. Sample quality control included individuals with heterozygosity values within three standard deviations (SD) from the mean, a missingness rate < 0.01, matching chromosomal and database-labelled sex and relatedness π-hat < 0.125. *SNPrelate* R package was used for principal component analysis (PCA) to calculate the first ten principal components to be added as covariates to control for population stratification in subsequent analysis.

The TRS-PRS were constructed using PRS-CS, a method that implements a high-dimensional Bayesian regression to perform a continuous shrinkage of SNP effect sizes using GWAS summary statistics and an external linkage disequilibrium (LD) reference panel (Ge et al., 2019). Summary statistics were obtained from the largest GWAS of TRS (Pardiñas et al., 2022). The LD reference panel was constructed using a European subsample of the UK Biobank (Bycroft et al., 2018). For the remaining parameters, the default options as implemented in PRS-CS were adopted.

2.3. GReX in the target population and GReX-RS calculation

We used PrediXcan (Gamazon et al., 2015) to impute GReX in our sample of FEP using the same prediction models (MASHR-based models) and brain tissues (PUT, SN, FL, HTH, HIP, CB, CAU, CNG and AMY) as in the TWAS analysis performed before. The analysis was performed following the standard procedure, as described by the authors (https://github.com/hakyimlab/PrediXcan).

GReX-RSs were estimated in our target sample of FEP using data from the S-PrediXcan TWAS on the TRS dataset (Pardiñas et al., 2022) as the discovery sample. The GReX-RS of each sample was the sum of the GReX of each gene weighted by its signed z-score value in the discovery sample (Rodriguez-López et al., 2020). Different GReX-RSs were estimated by selecting genes according to several P-value thresholds of the association in the discovery sample: 0.001, 0.05, 0.1, 0.5, and 1.

Genes included in the GReX-RS that were significantly associated with clinical data were tested for protein–protein interactions (PPi) using STRING v12.0, considering medium confidence interactions based on experiments and databases (Szklarczyk et al., 2023). A gene set enrichment analysis using the Biological Processes of the Gene Ontology databases was performed using a Kolmogorov–Smirnov test and corrected using the False Discovery Rate method as implemented in STRING v12.0 (Szklarczyk et al., 2023).

2.4. Statistical analysis

Data were analysed using SPSS 20.0 (statistical analysis software, IBM, Chicago, IL, USA). Two-tailed p-values < 0.05 were considered to be of statistical significance. Means and standard deviations were computed for continuous variables. The normality of continuous variables was tested using the Shapiro–Wilk tests. Correlations between GReX-RSs constructed at different p-value thresholds or for different brain tissues were assessed using Pearson's correlation coefficients. The significance of the GReX-RS in relation to clinical and cognitive data was evaluated by linear regression using sex, age, DUP, CPZ equivalents and 10 principal components of genetic PCA as covariates. Model fit was estimated as the increase in adjusted pseudo-R2. To assess the stability and reliability of regression estimates and their associated uncertainty, bootstrapping was used as a cross-validation approach and the confidence intervals were estimated using 1000 random samples. Multiple testing corrections were applied using Holm-Bonferroni sequential correction. According to this method, a p-value < 0.001 was considered statistically significant.

3. Results

We applied S-PrediXcan to TRS GWAS summary statistics. As shown in Fig. 2, no GReX showed significant associations with TRS in any of the nine tissues analyzed (complete results in Supplementary **Table S1**).

Table 1 shows the demographic and clinical characteristics of the target sample. GReX-RS was calculated for each individual in the target dataset as the sum of predicted expression of all genes weighted by its signed z-score in the discovery sample. Different p-value thresholds in the discovery sample were considered for gene inclusion in the GReX-RS model. As expected, GReX-RSs computed at different thresholds for each tissue showed strong correlations with each other and among the different tissues (**Supplementary Figue S1**). This correlation arises from the shared genetic regulation of gene expression between tissues, impacted by common genetic variants, and the presence of conserved regulatory elements across different tissues.. It is crucial to emphasize that while high correlation is often observed, it does not imply identical imputed gene expression across tissues. Tissue-specific nuances and differences persist, and the correlation primarily reflects the shared genetic factors influencing gene expression.

Each GReX-RS was tested for association with clinical data using linear regression models adjusted by sex, age at onset, DUP, CPZ equivalents and the first 10 components of the genetic PCA. Clinical data included: symptomatology (positive and negative Marder factors), functionality (FAST scale) at baseline and 1-year follow-up, and cognitive performance (sustained attention, verbal learning and memory, working memory, executive function and composite cognitive score) at 2-month follow-up (Supplementary **Table S2**).

GReX-RSs constructed with the SN, HIP and AMY at a p-value = 0.5 were significantly ($p<0.001$ according to Holm-Bonferroni sequential correction method) associated (SN: $R^2=0.061$, $F(3197)=4.25$, $p<0.001$; HIP: $R^2=0.088$, $F(3197)=6.33$, $p<0.001$; AMY: $R^2=0.098$, $F(3197)=7.14$, $p<0.001$) and significantly predicted (SN: $\beta=0.15$, 95 % CI=

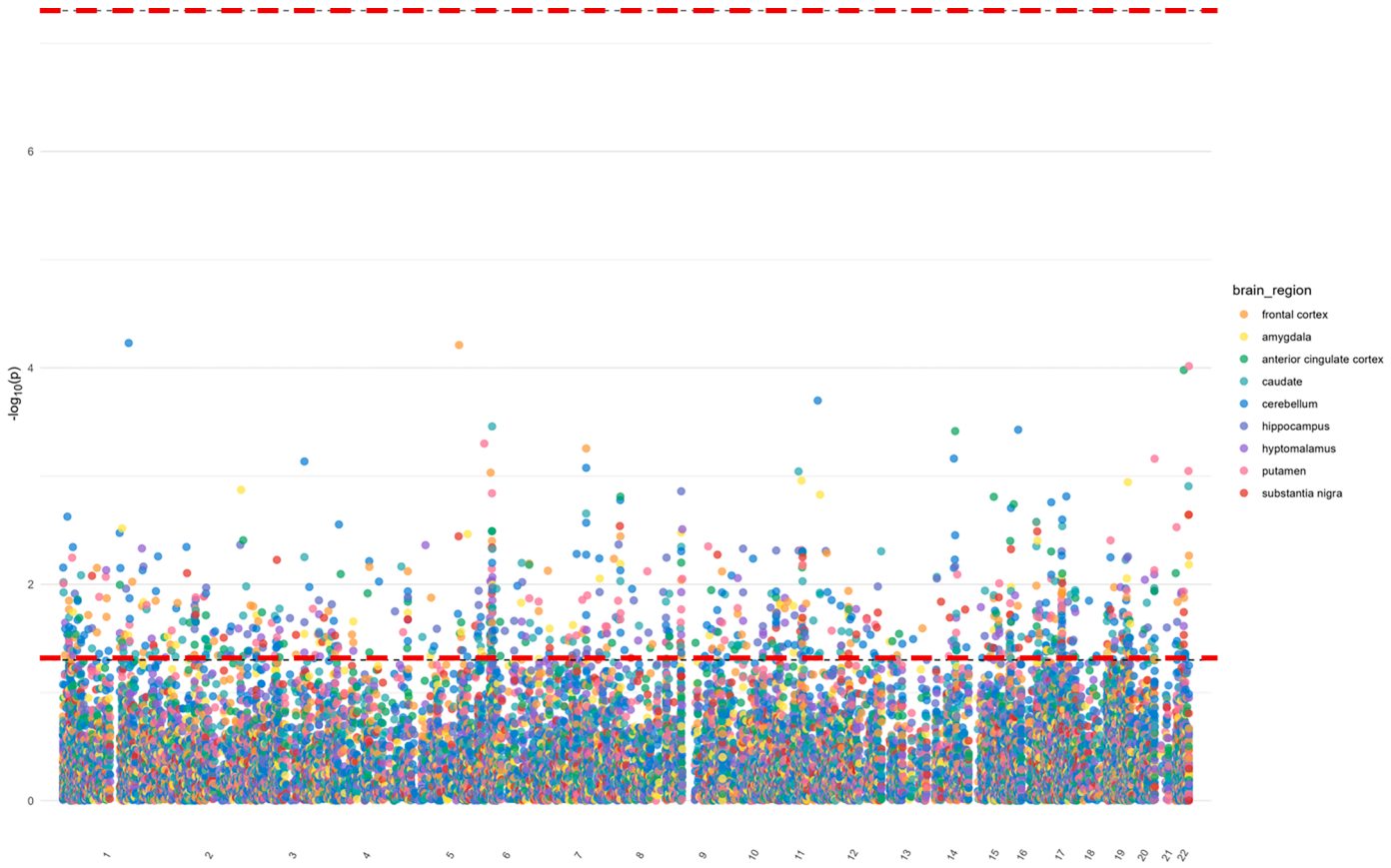


Fig. 2. Manhattan plot of the TWAS of TRS performed in the discovery sample. Results of the nine S-PrediXcan models based on brain tissues from GTEx v8 are shown. Dotted red lines indicate nominal significant thresholds of *p*-value = 0.05 and genome wide significant threshold of p-value = 5×10^{-8}.

Table 1
Main sociodemographic, cognitive, functional and clinical features of the 254 FEP patients of the target population.

Sociodemographic variables (Mean ± SD or n (%))	
Sex (Male/Female)	178(70)/76(30)
Age (years)	23.8 ± 6.1
Duration of untreated psychosis (days)	103.7 ± 126.7
Cognitive variables at 2-month follow-up (Mean±SD)	
Sustained Attention	88.6 ± 8.9
Working Memory	79.3 ± 16.2
Verbal Memory	136.1 ± 49.9
Executive Function	126.1 ± 42.7
Composite Score	295.9 ± 49.9
Clinical and functional variables at baseline (Mean±SD)	
Chlorpromazine equivalents	565.3 ± 469.3
Positive Marder PANSS Factor	21.9 ± 8.8
Negative Marder PANSS Factor	17.9 ± 8.1
Functionality (FAST)	27.5 ± 16.2
Clinical and functional variables at 1-year follow-up (Mean±SD)	
Chlorpromazine equivalents	287.8 ± 314.4
Positive Marder PANSS Factor	12.8 ± 5.6
Negative Marder PANSS Factor	14.1 ± 6.4
Functionality (FAST)	18.5 ± 14.4

FAST, Functioning Assessment Short Test; PANSS, Positive and Negative Syndrome Scale. .

[0.07:0.22], $p<0.001$; HIP: β=0.13, 95 % CI=[0.07:0.19], $p<0.001$; HIP: β=0.15, 95 % CI=[0.09:0.22], $p<0.001$) the severity of negative symptoms at 1-year follow-up (Fig. 3**a**). The FL score calculated at the 0.5 threshold explained 11.4 % of the variability (F(3180)=7.69, $p>0.001$) and was significantly associated with more cognitive impairments in sustained attention (β=−0.13, 95 % CI=[−0.20:−0.05], $p<0.001$) (Fig. 3**b**). The p-value of 0.5 was selected because it explained the largest proportion of variability in all cases (Fig. 3**c**).

As a sensitivity analysis we tested the association between the TRS-PRS and the clinical predictors of TRS using linear regression models adjusted by sex, age at onset, DUP, CPZ equivalents and the first 10 components of the genetic PCA. Non-significant associations were observed with positive (Basal: F(3, 249)=0.34, $p = 0.792$; 1-year: F(3 198)=0.41, $p = 0.966$) or negative symptomatology (Basal: F(3, 249)= 0.34, $p = 0.794$; 1-year: F(3 199)=2.21, $p = 0.08$), functionality (Basal: F (3, 199)=0.57, $p = 0.630$; 1-year: F(3 198)=1.75, $p = 0.158$) or cognitive performance (Attention, F(3, 180)=0.35, $p = 0.792$; Verbal memory, F(3, 194)=0.57, $p = 0.634$; Working memory, F(3, 202)=0.66, $p = 0.579$; Executive function F(3, 192)=0.33, $p = 0.802$; and Composite cognitive score, F(3, 174)=0.57, $p = 0.633$).

In comparison with random sets of proteins of similar size from the genome, the PPI networks built with the lists of genes included in the GReX-RS, (using p-values=0.5) which were significantly associated with clinical data (SN 814 genes, HIP 887 genes, AMY 1155 genes and FL 1112 genes), showed significantly more connections than expected ($p = 1.9\times10^{-5}$; 1.9×10^{-3}; 1.8×10^{-6}; 1.0×10^{-16}; respectively) (**Supplementary Figure S2**). A cluster analysis identified two functional clusters in the SN network, cluster 1 enriched with biological processes related to the immune system and the major histocompatibility complex II, and cluster 2 enriched with mitochondrial gene expression and translation and RNA metabolic processes (Fig. 4**a**). The HIP network included three clusters: cluster 1 enriched with cellular metabolic processes (including nitrogen compounds, RNA, DNA and proteins) and response to stress, cluster 2 enriched with transport and immune system processes, and cluster 3 enriched with acid metabolic processes (Fig. 4**b**). Two clusters

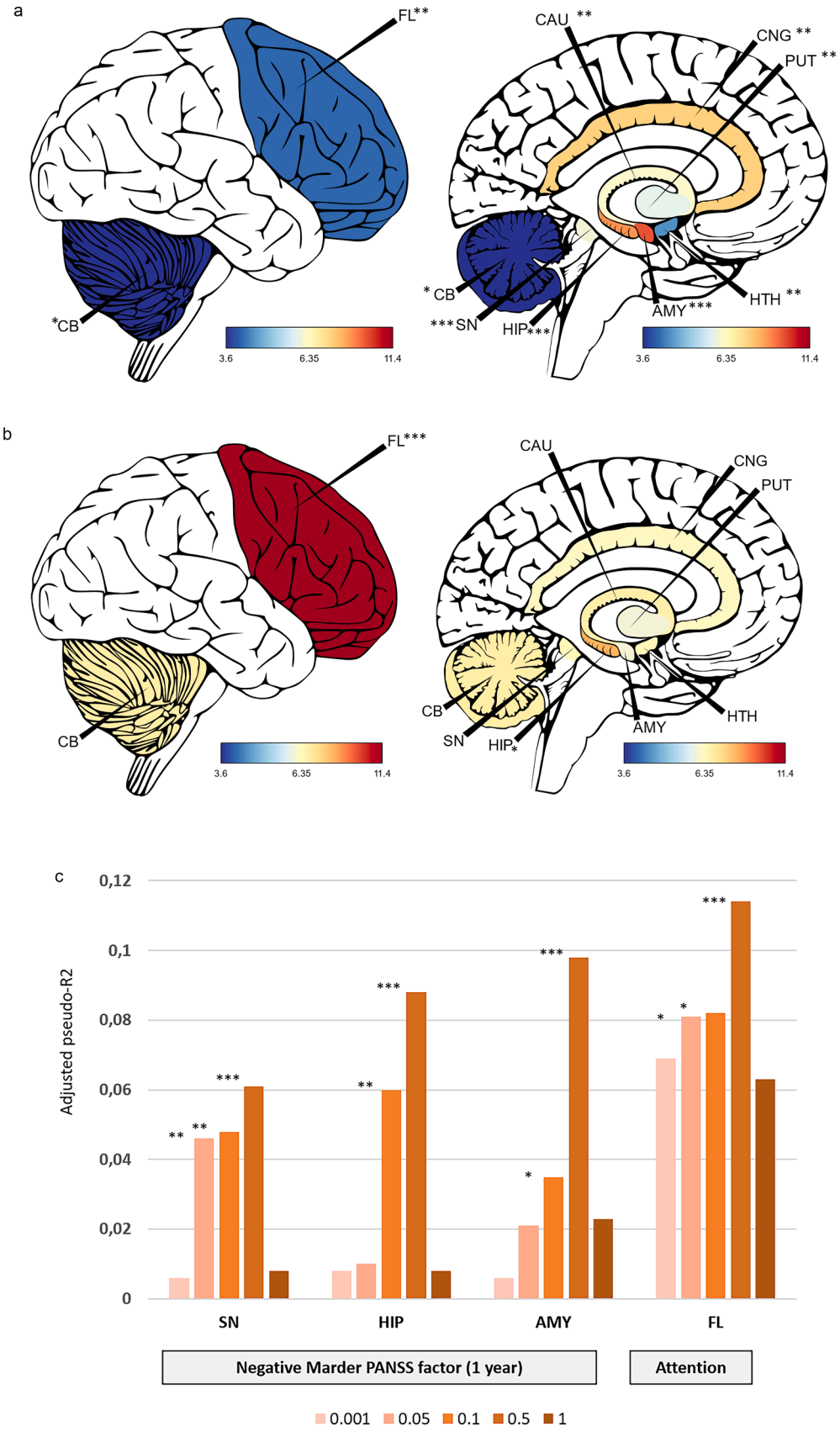


Fig. 3. (a) Explained variance (as the adjusted pseudo-R2) of the Negative Marder PANSS factor at 1-year follow-up for each brain region. (b) Explained variance (as the adjusted pseudo-R2) of the attention domain of cognition for each brain region. (c) Association between the SN, HIP, AMY and FL GReX-RS and the Negative Marder PANSS factor at 1-year follow-up and the attention domain of cognition. The y-axis shows the variance explained (as the adjusted pseudo-R2) by the GReX-RS at different p-value thresholds shown on the x-axis. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

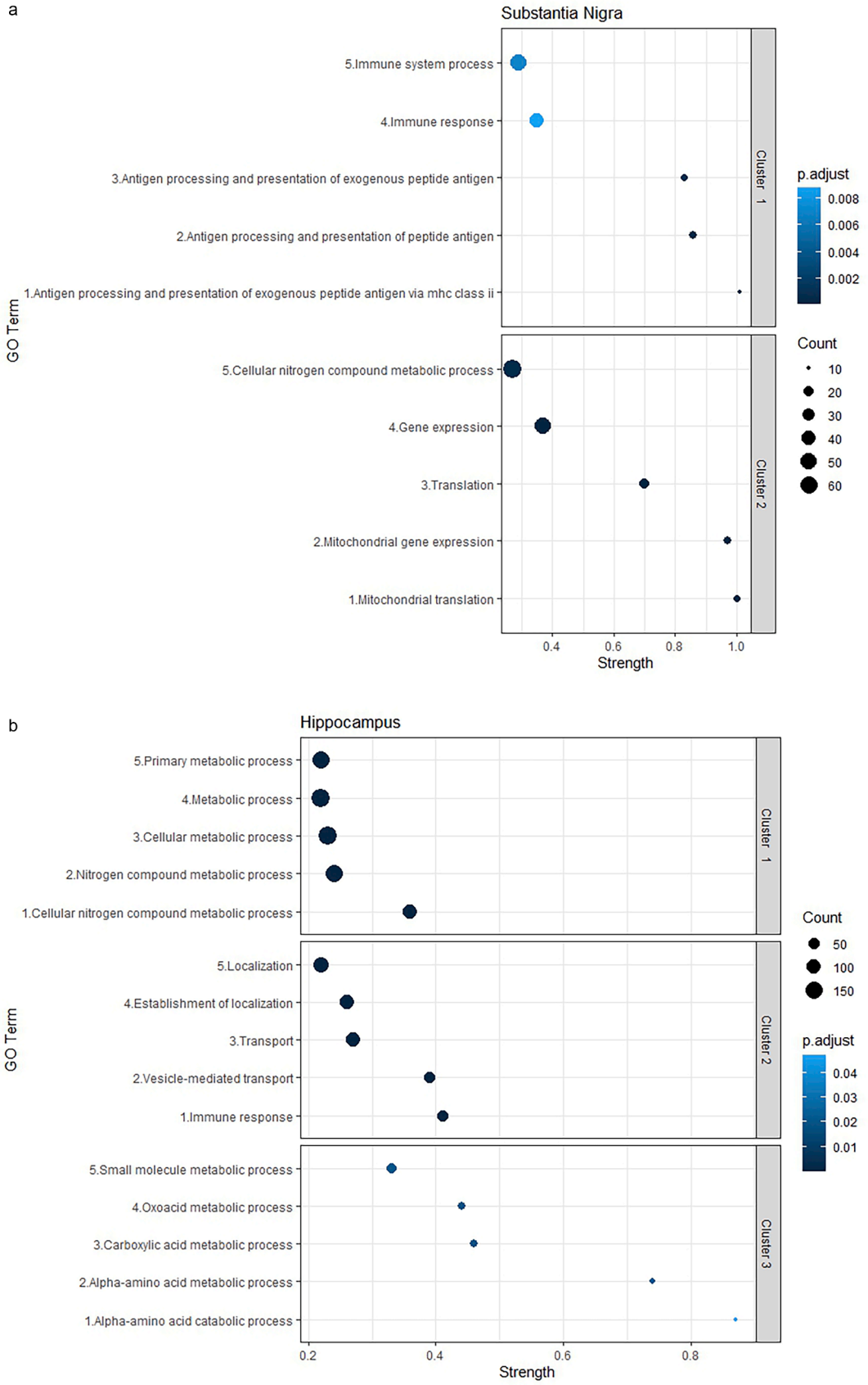
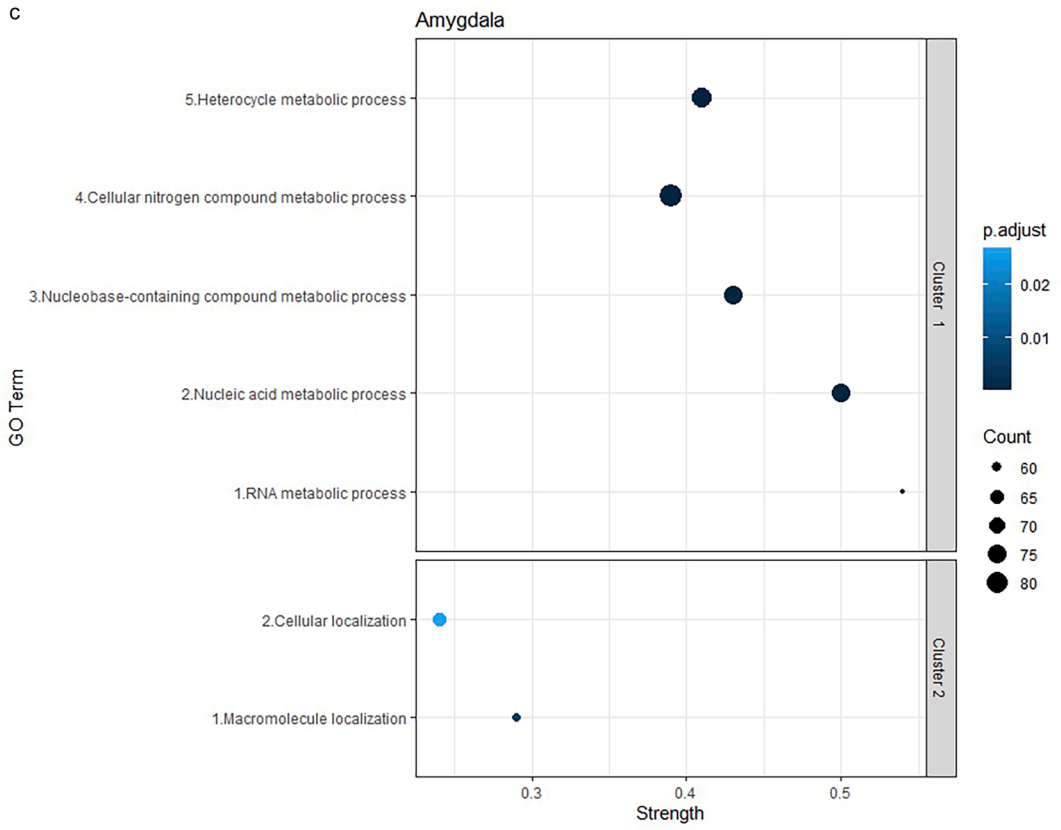


Fig. 4. Top five significant terms of the Biological Processes enrichment analysis of the Protein–Protein Interaction networks of (**a**) Substantia Nigra, (**b**) Hippocampus, (**c**) Amygdala and (**d**) Prefrontal Cortex. Counts refers to the number of genes in the network included in each biological process. Strength is a ranking metric for the enrichment analysis. .



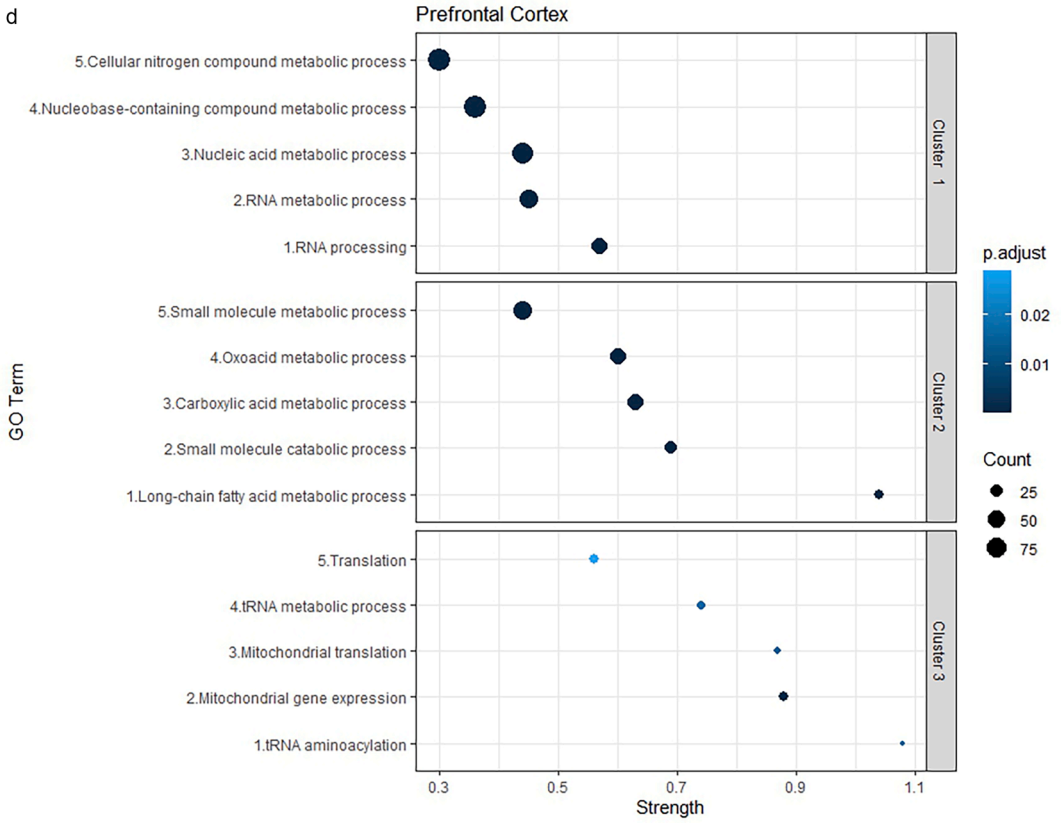


Fig. 4. (*continued*).

were identified in the network constructed with AMY genes: cluster 1 enriched in nucleic acid processes and cluster 2 enriched in cellular localization processes (Fig. 4**c**). Finally, three clusters were defined in the FL network: cluster 1 enriched in nucleic acid processes, cluster 2 enriched in acid and lipid metabolic processes and cluster 3 enriched in mitochondrial gene expression (Fig. 4**c**) (Supplementary **Table S3**).

4. Discussion

Our analysis provides evidence that the polygenic basis of TRS includes genetic variants that modulate the expression of certain genes in certain brain areas (SN, HIP, AMY and FL), which at the same time are related to clinical features in FEP patients, mainly persistence of negative symptoms and cognitive alterations in sustained attention, which have also been suggested as clinical predictors of TRS. Although we cannot infer causality, our results provide biological and anatomical substrates as well as a clinical interpretation of the genetic association with TRS. Moreover, the identified brain areas and the clinical symptoms associated are consistent with the neurobiological basis of TRS (Wada et al., 2022; Yang et al., 2022) and add more evidence to the idea that TRS is categorically a different schizophrenia subtype, with distinct neurobiological foundations from treatment responsiveness schizophrenia (Gillespie et al., 2017; Pardiñas et al., 2022). The lack of significant associations between the constructed TRS-PRS and clinical data support the superiority of GReX scores in capturing relevant information compared to conventional PGS in the context of TRS.

We first performed a TRS TWAS using the summary statistics of the largest TRS GWAS performed to date (Pardiñas et al., 2022). None of the GReX imputed in the nine brain areas considered in our study showed a significant association with TRS. In the original TRS GWAS no genetic variant achieved significance, but the constructed TRS-PRS was significantly associated with TRS (Pardiñas et al., 2022). Moreover, the TRS-PRS showed a strong overlap with cognitive PRs, and in our first-episode sample, the constructed GReX-RS in the FL was significantly associated with the cognitive domain of attention in a sample of FEP.

The significant associations of GReX-RSs with negative symptoms and cognitive deficits in our FEP cohort are consistent with the results of previous clinical studies of TRS. In these studies, higher severity and/or persistence of negative symptoms and worse cognitive performance were identified as core symptoms of TRS (de Bartolomeis et al., 2018; Demjaha et al., 2017; Frydecka et al., 2016; Kowalec et al., 2021; Malaspina et al., 2000; Millgate et al., 2022; Robinson et al., 1999; Yang et al., 2022). The notion that higher negative symptomatology and poorer cognitive performance, but not positive symptomatology, predict a worse response to treatment is understandable, since antipsychotics are mainly effective in the treatment of positive symptoms, and less in the treatment of negative and cognitive symptoms. Thus, so far, progress in the development of innovative treatments has been slow and negative symptoms often represent an unmet need (Galderisi et al., 2021). In this context, it is also worth highlighting the need to take into consideration those negative symptoms that are considered to be secondary to treatment-resistant positive symptoms and those considered primary negative symptoms (deficit schizophrenia), which are persistent throughout the course of the illness, and respond poorly to antipsychotic treatments. This primary and enduring symptomatology is associated with greater impairment of neurocognitive abilities, poorer response to treatment and worse outcomes. Thus, reduced general cognitive abilities in schizophrenia patients with primary negative symptoms might also be related to a neurodevelopmental trajectory, with early cognitive impairment interfering with the acquisition of subsequent competences and, consequently, having a more severe impact on long-term functional outcome (Galderisi et al., 2018).

It's remarkable that significant associations related to TRS were observed in a cohort of FEP patients at the one-year follow-up. This suggests that studying the progression of the disease over time, rather than just assessing patients at the initial episode, can provide valuable insights into TRS. Longitudinal assessment in cohorts of FEP patients offers several advantages, mainly the study of the natural clinical progression of the disease without the confounding effects of chronic treatments. FEP cohorts present a unique opportunity to identify predictors of early treatment resistance. By tracking patients from their first episode, we can potentially identify factors or markers that indicate a higher risk of developing TRS. This information could be crucial for early intervention and personalized treatment approaches. The fact that significant associations were observed at the follow-up assessment but not at the baseline is an intriguing finding. It suggests that it is the presence of persistent negative symptoms and cognitive impairment over time that increases the risk of TRS, rather than the severity of these symptoms at the initial episode. This insight may have implications for the timing and nature of interventions aimed at preventing or managing TRS. During the acute phase of FEP, patients may exhibit a high degree of symptom severity, which could make it challenging to stratify or identify specific risk factors for TRS at that early stage. This highlights the importance of observing patients over a longer period to distinguish between transient and persistent symptoms.

The brain areas identified (SN, HIP, AMY and FL) have been repeatedly associated with TRS (Anderson et al., 2015; Huang et al., 2020; Kim et al., 2020; Liu et al., 2022; McNabb et al., 2018; Wannan et al., 2019; White et al., 2016; Zugman et al., 2013) but also with negative symptoms and cognitive performance (Huang et al., 2022; Millan et al., 2014; Prestia et al., 2015; Rahm et al., 2015; Toll et al., 2022). These brain areas are involved in limbic loops of the cortico-thalamic circuits that have been implicated in both TRS and negative and cognitive symptoms of schizophrenia (Chen et al., 2019; Kim et al., 2022; Roldán et al., 2020; Wada et al., 2022; Zhu et al., 2022). These circuits involve dopamine and glutamate neurotransmission. Several findings suggest that glutamatergic dysfunction in the FL may be located temporally and spatially upstream of dopaminergic dysfunction in the SN and striatum, through the regulation of HIP and AMY activity via thalamic circuits (Wada et al., 2022).

Overall, our results are consistent with the different neurobiological hypotheses of TRS, which may converge and are not mutually exclusive. Several lines of evidence point to dopamine and glutamate dysfunction in the development of TRS, although there is some evidence that neuroinflammatory processes could also play a role (Potkin et al., 2020). The dopamine supersensitivity hypothesis explains TRS in patients who were responsive to antipsychotic treatment at illness onset. This theory proposes that continuous blockade of dopamine receptors by antipsychotic medications leads to dopamine supersensitivity, causing TRS. However, this hypothesis is not supported by clinical data (Potkin et al., 2020). Another dopamine hypothesis for TRS proposes that spontaneous hyperactivation of dopamine function is a prerequisite for antipsychotics to be effective, while the emergence of psychotic symptoms heavily depends on phasic firing of dopamine neurons. Given that TRS may have lower dopamine synthesis (Nakata et al., 2017), the effects of antipsychotics are insufficient to elevate dopamine activity to the point of blocking depolarization, and are thus limited in efficacy in TRS patients (Wada et al., 2022). In this context, some authors have also proposed that, since the dopamine system is the primary target of all current antipsychotic treatments, primary TRS may be related to dysfunction in neurotransmitters other than dopamine (Correll and Howes, 2021).

There is evidence that abnormalities in glutamate regulation may specifically play a role in TRS. Neuroimaging studies showed that glutamate levels in several brain regions were higher in patients with TRS compared with healthy controls or patients with schizophrenia who were treatment responsive (Demjaha et al., 2014; Goldstein et al., 2015; Mouchlianitis et al., 2016; Nakahara et al., 2022). Indirect evidence for glutamate's role in clozapine-responsive TRS is supported by studies examining the effect of clozapine on the glutamatergic system (Fukuyama et al., 2019; Tanahashi et al., 2012). Some evidence suggests that TRS could be related to neuroinflammatory processes affecting

synaptic pruning (Jiao et al., 2022). Consistent with this suggestion, the genes included in the GReX-RSs that were significantly associated with negative symptoms were enriched in biological processes related to the immune system.

The results of our study should be considered in the context of several limitations. First, one key limitation is the lack of a TRS diagnosis in our FEP cohort. Although we explored the association of GReX-RSs with clinical predictors of TRS we were not able to explore whether these clinical variables, and other well-known predictors of TRS such as gender, age at onset, DUP or adherence, were truly predictors of TRS in our sample. Second, several constraints are associated with the use of the PANSS as it was not designed with the purpose of solely measuring negative symptoms. To account for this, we used the PANSS-Marder Factor Scores, which apply stricter criteria for assessing positive and negative symptomatology. Third, the limited sample size may have reduced the statistical power and the ability to detect small effects. Further research with larger sample sizes is therefore required. We also have to consider the limited statistical power of the original GWAS used in our study and its possible effect on the construction of the GReX-RS. Finally, the short follow-up period is a potential limitation in this study. Nonetheless, the study is a naturalistic and multicentric study based on the entire Spanish population, and comprises the largest and best characterized first-episode sample of the country. Additionally, the GReX-RSs were calculated from the largest TRS-GWAS and therefore the genetic variants identified have good potential to capture the genetic susceptibility of the phenotypes explored.

Our results provide a clinical explanation of the polygenic architecture of TRS. The characterization of the clinical and cognitive phenotypes associated with TRS gene expression in specific brain regions can give us insight into the biological mechanisms underlying TRS, and therefore how to diagnose this complex phenotype and improve its prevention and treatment from the earliest stages of the illness. Understanding gene expression patterns in TRS and the brain areas involved will contribute to the study of potential pathways and ultimately help improve psychiatric classification tools in personalized medicine. In this regard, these results highlight the usefulness of integrating results from large genetic studies with well-controlled clinical samples with longitudinal phenotypes to explore the biological and clinical basis of TRS.

Data availability statement

The clinical data that support the findings of this study are not openly available due to contain human data and are available from the corresponding author upon reasonable request.

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Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Author statement

LLP and NR performed the preprocessing of the data and the statistical analysis, and wrote the first draft of the manuscript. AGS and AGassisted in the preprocessing of the data. AMP, DOB, IM, GM and PG assisted in the statistical analysis and participated in the drafting of the manuscript and figures. MP, MJC, MB participated in the selection, acquisition and preparation of the samples. SM was the responsible of the conception and design of the study, the acquisition of funding and the supervision of the statistical analysis.

CRediT authorship contribution statement

Llucia Prohens: Conceptualization, Formal analysis, Writing – original draft. **Natalia Rodríguez:** Data curation, Investigation. **Àlex-Gonzàlez Segura:** Data curation, Formal analysis. **Albert Martínez-Pinteño:** Data curation. **David Olivares-Berjaga:** Data curation, Software. **Irene Martínez:** Methodology. **Aitor González:** Methodology, Software. **Gisela Mezquida:** Data curation. **Mara Parellada:** Data curation. **Manuel J Cuesta:** Data curation. **Miquel Bernardo:** Data curation, Funding acquisition. **Patricia Gassó:** Data curation, Formal analysis. **Sergi Mas:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

M. Bernardo has been a consultant for, received grant/research support and honoraria from, and been on the speakers/advisory board of ABBiotics, Adamed, Angelini, Casen Recordati, Janssen-Cilag, Menarini, Rovi and Takeda. The rest of the authors reported no biomedical financial interests or potential conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2024.115722.

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