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CX3CR1 is dysregulated in blood and brain from schizophrenia patients

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Abstract

The molecular mechanisms underlying schizophrenia remain largely unknown. Although schizophrenia is a mental disorder, there is increasing evidence to indicate that inflammatory processes driven by diverse environmental factors play a significant role in its development. With gene expression studies having been conducted across a variety of sample types, e.g. blood and postmortem brain, it is possible to investigate convergent signatures that may reveal interactions between the immune and nervous systems in schizophrenia pathophysiology. We conducted two meta-analyses of schizophrenia microarray gene expression data (N=474) and non-psychiatric control (N=485) data from postmortem brain and blood. Then, we assessed whether significantly dysregulated genes in schizophrenia could be shared between blood and brain. To validate our findings, we selected a top gene candidate and analyzed its expression by RT-qPCR in a cohort of schizophrenia subjects stabilized by atypical antipsychotic monotherapy (N=29) and matched controls (N=31). Meta-analyses highlighted inflammation as the major biological process associated with schizophrenia and that the chemokine receptor *CX3CR1* was significantly down-regulated in schizophrenia. This differential expression was also confirmed in our validation cohort. Given both the recent data demonstrating selective CX3CR1 expression in subsets of neuroimmune cells, as well as behavioral and neuropathological observations of CX3CR1 deficiency in mouse models, our results of reduced CX3CR1 expression adds further support for a role played by monocyte/microglia in the neurodevelopment of schizophrenia.

Keywords

CX3CR1 • schizophrenia • mRNA• inflammation • transcriptomics •meta-analysis

1. Introduction

Schizophrenia (SCZ) is a complex and devastating brain disorder with an unknown etiology. Although heritability for SCZ is estimated to be close to 70 % (Lichtenstein et al., 2009; Sullivan et al., 2003), extensive investigations over the past two decades to define conserved genetic variations among thousands of SCZ samples has not led to definitive results (Gratten et al., 2014). Gene expression profiling has been proposed as an alternative strategy to identify the possible causes of the disease and to understand gene-environment interactions (Mitchell and Mirnics, 2012). The transcriptional landscape can be considered an intermediate phenotype between genomic sequence variability and complex traits that may help to reveal disruptions to biological pathways that underlie the progressive decline from normality to a psychiatric pathology. In fact, the high throughput transcriptome profiling experiments conducted with DNA microarrays identified several molecular processes in SCZ such as myelination, synaptic transmission, metabolism, ubiquitination and immune function (Kumarasinghe et al., 2012; Mistry et al., 2013b). Historically, as for all investigated psychiatric diseases, postmortem brain samples have been considered as the gold standard material to profile SCZ (Arion et al., 2007; Barnes et al., 2011; Chen et al., 2013; Hagihara et al., 2014; Mistry et al., 2013a, b; Perez-Santiago et al., 2012; Saetre et al., 2007; Schmitt et al., 2011; Torkamani et al., 2010). However, such tissue presents many limitations including access and collection of large sample sizes, tissue preparation and conservation, and antemortem diagnosis. With the aim of developing biomarkers, blood samples have been increasingly utilized because they are easily obtained and allow longitudinal follow-up of gene expression some of which is also correlated in brain tissue (Mamdani et al., 2013; Woelk et al., 2011).

Many transcriptomic microarray studies in SCZ have been made available in public domains such as the Gene Expression Omnibus (GEO) from NCBI (Barrett et al., 2013), ArrayExpress from EBI (Rustici et al., 2013), and the Stanley Medical Research Institute (SMRI) online genomics database (Higgs et al., 2006). Raw data from both postmortem and blood SCZ studies can be easily retrieved from these databases for use in a meta-analysis with enough sample size and sensitivity for the identification of differentially expressed genes and biological processes. With increasing numbers of analyzed samples, it is important to apply normalization procedures that will balance effects that may arise from the heterogeneity in tissue regions, microarray platform, and sample quality that could collectively deteriorate the meta-analysis. Indeed, different methods have been proposed and discussed to conduct meta-analysis (Chang et al., 2013; Chen et al., 2011; Conlon et al., 2007; Lopez et al., 2008; Phan et al., 2012; Schurmann et al., 2012; Seita et al., 2012; Stevens and Doerge, 2005; Tian and Suarez-Farinas, 2013; Warnat et al., 2005).

In the present study, we conducted a meta-analysis to explore whether a common gene expression profile exists across various brain regions that is shared with blood samples and distinguishes SCZ from healthy individuals. To achieve this goal, we used microarray data from SCZ and matched control cohorts publicly available sources, as well as data made available from the Gardiner et al. (2013), and Kumarasinghe et al. (2013) publications (Gardiner et al., 2013; Kumarasinghe et al., 2013). To validate our analysis, we tested the expression of a gene candidate on a cohort of stabilized SCZ patients and healthy controls by RT-qPCR. Taken together, our work pinpoints a biological process and potentially specific cell populations for future experiments investigating SCZ pathophysiology.

2. Materials and methods

2.1 Microarray Datasets

Microarray datasets were selected on the basis of whether they used either postmortem brain or blood tissue (i.e. cell lines were excluded), the availability of raw data, and information on covariates (i.e. age and sex for blood samples, as well as pH and postmortem interval (PMI) for brain tissues). The treatment status and the acute or remitted status of patients were not taken into account for the selection of datasets. Each dataset was comprised of neuropathologically normal and SCZ subjects, as diagnosed and reported in their respective studies (Table 1). Sources for data include GEO (http://www.ncbi.nlm.nih.gov/geo), ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), and the Stanley database (https://www.stanleygenomics.org/). GEO and ArrayExpress studies were identified by extensive manual and combinatorial keyword searches (schizophrenia, psychosis, control, human, brain, blood, microarray) until November 1st 2014. Although additional datasets were retrievable (including some microarrays with custom designs that did not cover the whole genome), many represented repeated runs of the samples from the same subjects, so datasets were selected that do not allow overlap between samples and/or individuals. Two additional studies of whole blood were obtained from the investigators (Gardiner et al., 2013; Kumarasinghe et al., 2013). In total, ten datasets represented various postmortem brain regions (prefrontal, frontal and temporal cortex, cerebellum, hippocampus, striatum and thalamus) and five utilized blood (whole blood and PBMCs) (see Table 1 for details of each data set).

2.2 Analysis of Microarray Data

After downloading the raw data, microarray analysis was conducted using an in-house pipeline inspired by Turnbull et al. and developed under the R statistical language (Turnbull et al., 2012). Using R/Bioconductor software (Gentleman et al., 2004), the signal intensities were normalized by the quantile method (limma package, http://www.bioconductor.org/packages/release/bioc/html/limma.html) (Smyth, 2005), with prior transformation using the RMA algorithm (affy package, http://www.bioconductor.org/packages/release/bioc/html/affy.html) exclusively for Affimetrix datasets (Gautier et al., 2004). Probes were assigned to gene symbols based on the annotation files provided by GEO. For genes with multiple expressed probe sets, mean expression intensity was calculated within each microarray dataset. Next, according to their Entrez ID annotation, genes in common between Affymetrix (postmortem samples) and Illumina (blood samples) platforms were selected to merge the datasets into one single postmortem dataset (8655 genes) and one single blood dataset (16 661 genes). Batch effects across the postmortem and blood datasets were removed using the methods implemented in the ComBat algorithm, that exploits variance moderation during data adjustment, using the sva R package (http://www.bioconductor.org/packages/release/bioc/html/sva.html) (Johnson et al., 2007). Differences between SCZ and control groups were assessed by analysis of variance (ANOVA) using the maanova R package (http://www.bioconductor.org/packages/release/bioc/html/maanova.html). The resulting p-values were further adjusted for multiple testing using the q-value method to control the false discovery rate (FDR) (Storey and Tibshirani, 2003).

2.3 Gene ontology analysis

Lists of genes with significant changes were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009) to identify statistically relevant biological pathways altered in SCZ using medium classification stringency, p-value < 0.05 and FDR ≤ 10%. A Venn diagram of meta-signatures was built using the jvenn tool (Bardou et al., 2014).

2.4 Validation cohort

Twenty-nine stabilized SCZ patients (treated with either risperidone or aripiprazole) and thirty-one healthy controls were part of a neuroimaging cohort study conducted in Marseille (France) with the aim to identify links between disease phenotype, gene expression and neuroimaging variables obtained after using a new functional magnetic resonance imaging (fMRI) paradigm (Comte et al., 2015; Comte et al., 2014). Only patients who met the Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision (DSM-IV-TR) criteria for SCZ were recruited for the study. A complete medical examination, including medical history and physical examination was performed on admission. Inclusion criteria were: being stabilized by atypical antipsychotic monotherapy for at least six weeks (i.e., same antipsychotic drug at the same dosage), meeting remission criteria (Andreasen et al., 2005), and having a normal general medical examination with no DSM-IV-TR Axis I disorder comorbidity or drug addiction. Patients were either hospitalized in a general public mental hospital or outpatients regularly followed by a psychiatrist.

The severity of the patients' symptoms was evaluated using the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987). Subscores from the PANSS were used to derive the following five-factor scores: negative, positive, cognitive/disorganization, excitation/hostility and anxiety/depression (Emsley et al., 2003). Additional clinical assessment included the Calgary Depression Scale for Schizophrenia (CDSS) (Addington et

al., 1993), and the State-Trait Anxiety Inventory (STAI) with its two subscales: the State Anxiety Scale (S-Anxiety) evaluating the current state of anxiety and the Trait Anxiety Scale (T-Anxiety) evaluating relatively stable aspects of anxiety proneness (Spielberger et al., 1983). Socio-demographic information, including age, gender and illness duration was collected, and tobacco smoking evaluated. The doses of antipsychotic medications were converted to chlorpromazine equivalence (Woods, 2003).

For the control group, participants were recruited through advertising in the local community of Marseille. Before entering the study, subjects underwent a medical interview and examination. The non-patient version of the Structured Clinical Interview for DSM-IV (SCID) was used to ensure the absence of psychiatric disorder and any psychiatric history (First et al., 2002). Participants had no current or past serious medical or neurological conditions and were not taking any psychotropic drugs at the time of the study. Tobacco use was also evaluated.

All experiments on human subjects were conducted in accordance to the latest version of the Declaration of Helsinki. The project was approved by the local ethics committee (Comité de Protection des Personnes, CPP Sud Méditerranée II, Marseille, France, study registered under ID RCB: 2009-017673-38) and written informed consent was obtained after a complete description of the study was given to the subjects. Subjects received financial compensation for their participation.

2.5 Blood mRNA extraction

Venous blood (8-10 ml) was collected from fasting patients and matched-controls in EDTA tubes between 8:30 - 9:00 a.m. and processed within two hours. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll density centrifugation.

Total RNA was extracted from the PBMCs with the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's procedure for total RNA isolation. RNA concentration was determined using a nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and all samples exhibited an RNA integrity number (RIN) above 8.

2.6 Real-time RT-PCR

RNA (1 μg) was reverse transcribed with the High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). cDNA was combined with a TaqMan® universal PCR Master Mix (Applied Biosystems) and PCR reactions were performed on an ABI PRISM 7900HT thermocycler according to the manufacturer's recommendations (Applied Biosystems). All PCR reactions were performed in triplicate. For *CX3CR1* mRNA expression, primer sets and probes were selected using the web portal of the manufacturer (Applied Biosystems: Hs00365842_m1). The relative fold change expression (FC) was calculated using the $2^{-\Delta\Delta Ct}$ formula with the DataAssist software (Applied Biosystems, v3.0). The differential expression was also compared to a calibrator sample; specifically to the mean of all control samples. The reference gene used for normalization was *G3BP2 (*Hs00907696_m1), a gene that exhibited very stable expression across PBMC samples from a previous longitudinal study of both controls and patients suffering from major depression (Belzeaux et al., 2012). *G3BP2* was also expressed at similar levels compared to the target gene *CX3CR1*.

2.7 Statistical Analysis in validation study

Skewness and Kurtosis were calculated for each continuous demographic and clinical variable to test for normality. Variables were compared between patients and matched-controls with a

chi-squared (χ^2) test for qualitative variables and ANOVA or t-test for quantitative variables. Gene expression differences between groups were analyzed using General Linear Models (GLMs) including groups as fixed factors and potential confounding factors as covariables. Confounding factors were defined if demonstrating a difference between groups with p-value < 0.2 in univariate analyses. Correlation between clinical dimension and gene expression were calculated using Pearson's correlation test. All statistical tests were conducted using the IBM SPSS Statistics v20 software. A p-value < 0.05 was considered as significant.

3. Results

3.1 Meta-analysis in postmortem brain

We first compiled a brain cohort from 204 SCZ patients and 212 normal controls by pooling data from 10 studies (Tables 1 and 2). Brain regions investigated included the prefrontal, frontal and temporal cortices, cerebellum, hippcampus, striatum and thalamus. Analysis of the demographic variables identified a significant difference in PMI and pH, but not age and gender between the SCZ subjects and controls (Table 2). After normalization and the removal of underexpressed probes and batch effect (see Methods for details), 8655 genes remained in common from the 10 datasets that were pooled from the postmortem brain dataset. ANOVA was carried out to identify differentially expressed genes in SCZ versus control after taking into account age, gender, pH and PMI as covariables. After correction for multiple testing, 264 genes displayed altered expression ($q \leq 1.0E-4$) equally distributed among down-regulated (N = 131, Supplementary Table 1) and up-regulated genes (N = 134, Supplementary Table 2). Ontological annotations with DAVID revealed that six groups of biological processes were overrepresented (FDR < 10%) in the brain gene list: organic substance response, stimulus/stress response, defense/inflammatory response, proteasome/ubiquitination/catabolism, energy production/mitochondrion, metallothionein (Table 3).

3.2 Meta-analysis in blood

A second blood cohort consisted of 240 SCZ patients and 238 healthy controls with similar age distribution but significant overrepresentation of men in the SCZ group (Tables 1 and 2). After raw signal processing and quality control measures, 16 661 expressed genes remained in common to the five datasets that were pooled for the blood dataset. ANOVA was performed

with age and gender added as covariables to address their influence on gene expression. To obtain a similar number of genes compared to the brain meta-analysis, we adjusted the threshold q-value to 0.010 and identified 132 and 123 genes that were down- and up-regulated in SCZ patients compared to controls, respectively. Ontological annotations with DAVID revealed that two groups of biological processes were overrepresented (FDR < 10%) in the blood gene list: wounding/defense response/inflammatory response/lymphocyte activation/cytokine binding and protein kinase cascade (Table 4).

3.3 Convergence of gene expression profiles in brain and blood

To explore whether a common meta-signature was shared between brain and blood samples, we drew a Venn diagram based on down- and up-regulated genes in brain ($q \leq 0.001$) and blood ($q \leq 0.05$) cohorts (the statistical thresholds have been adjusted to compare approximately the same number of genes between tissues) (Figure 1). Out of the 1247 dysregulated genes in total, 13 genes were differentially expressed in the same direction in both brain and blood datasets. Of these, 10 genes (*ABCF1* and *CX3CR1* down-regulated; *ADM*, *BCL6*, *LCN2*, *NCF4*, *S100A8*, *S100A12*, *TSPO*, and *TLR2* upregulated) have roles in inflammation, immunity and wound responses. Indeed, a comparison of Tables 3 and 4 demonstrates that the brain and blood datasets share in common dysregulation of the defense/wounding/inflammatory response pathways from which we highlighted 3 genes present in our meta-signature: *ADM*, *S100A8* and *CX3CR1*. Since *ADM* and *S100A8* had already been validated as up-regulated in SCZ (Kakiuchi et al., 2008; Maycox et al., 2009; Perez-Santiago et al., 2012), we decided to focus further validation on the chemokine/fractalkine receptor, *CX3CR1*. In fact, this gene has an important role in the activation of the immune response and characterizes cells of myeloid origin where its expression is highly dependent upon the microenvironment and pathological context.

3.4 CX3CR1 expression in a validation cohort

To further validate the meta-signature, we explored *CX3CR1* gene expression by RT-qPCR on PBMCs from a validation cohort of remitted SCZ and non-psychiatric control subjects (Table 5). After verifying that all continuous variables were normally distributed, we observed a significant down-regulation of *CX3CR1* expression in SCZ compared to controls (FC = -1.08; p = 0.025), in agreement with the blood meta-analysis. Tobacco smoking is a confounding factor that distinguished SCZ from controls in the validation cohort (Table 5). We tested this covariable in a general linear model and confirmed that down-regulation of *CX3CR1* expression remained significant in SCZ subjects compared to controls (p = 0.021). Age and gender could introduce some bias in the blood meta-analysis, so these variables were also included in the *CX3CR1* gene expression analysis, though the differential expression remained significant (p = 0.018). *CX3CR1* mRNA expression could be influenced by antipsychotic medication since our patients were either on risperidone or aripiprazole monotherapy, but this association was not significant (p = 0.363). Since clinical data was collected for the validation cohort, it was assessed whether any clinical dimension was correlated with *CX3CR1* expression within SCZ patients. Interestingly, we identified a significant correlation of *CX3CR1* expression with the depression-anxiety dimension of the 5-dimension PANSS (r = -0.36; p = 0.048) and with the general psychopathology score of the classical 3-dimension PANSS (r = -0.46; p = 0.012). Furthermore, we observed a strong correlation between *CX3CR1* expression and the score of the CDSS (r = -0.632; p = 0.00023) as well as with both STAI subscales: S-Anxiety (r = -0.301; p = 0.029) and T-Anxiety (r = -0.354; p = 0.009).

4. Discussion

This study has presented for the first time a large-scale evaluation of the consistency of overlap between peripheral blood and postmortem brain gene expression in SCZ with the aim to discover transcriptional biomarkers of the disorder. A decade ago, Glatt et al. (2005) were the first to compare the gene expression profiles of blood and brain. Although they used a small cohort of SCZ cases and controls, their pioneering work has paved the way for using gene expression to identify biomarkers in psychiatric diseases (Glatt et al., 2005). In the present study, we conducted two meta-analyses in blood and brain using the raw data of 15 microarray studies to investigate gene expression from nearly 1000 subjects, equally divided between SCZ patients and non-psychiatric controls. To our knowledge, no meta-analysis has been previously conducted on blood microarray data from SCZ, although several studies had combined data from two to seven human postmortem brain tissue microarray studies (Hagihara et al., 2014; Logotheti et al., 2013; Mistry et al., 2013a, b; Perez-Santiago et al., 2012; Torkamani et al., 2010). Gene expression network analyses, which describe the interactions among groups of transcripts, demonstrated well-preserved membership and connectivity of case modules relative to control modules (Chen et al., 2013; Torkamani et al., 2010). Although SCZ has been viewed, mainly through imaging studies, as a disorder of reduced functional and structural cortical connectivity, it is proposed that this is not driven by differences in underlying molecular connectivities but rather from altered expression levels of genes with roles in important biological processes. The search for biological processes that are altered in SCZ has consistently revealed those involved in neuron development (Chen et al., 2013; Torkamani et al., 2010), metallothioneins (Chen et al., 2013; Logotheti et al., 2013; Perez-Santiago et al., 2012), energy metabolism (Hagihara et al., 2014; Mistry et al., 2013a, b; Torkamani et al., 2010), ubiquitination (Mistry et al., 2013a, b), and defense/immune/inflammatory response (Logotheti et al., 2013; Mistry et al., 2013a, b).

Importantly, the 264 genes that were dysregulated in our brain meta-analysis (q-value < 1.0E-4) confirmed the involvement of the last four biological processes that were also enriched in other microarray studies (Altar et al., 2005; Arion et al., 2007; Iwamoto et al., 2005; Saetre et al., 2007; Schmitt et al., 2011) (see methods). This was further supported by results from recent next generation sequencing investigations (Fillman et al., 2013; Hwang et al., 2013), that were not included in this report.

To define a list of dysregulated genes in brain samples, we have been careful to take into account not only the effects of age and gender (even if not significantly different between cases and controls), but also pH and PMI that differ between control and SCZ samples (Table 2). Of note, the significantly reduced brain pH observed in SCZ samples was previously suggested as a possible consequence of alterations in energy metabolism mentioned above (Iwamoto et al., 2005; Iwamoto and Kato, 2006; Perez-Santiago et al., 2012; Prabakaran et al., 2004). Unfortunately, in the brain cohort, we lacked information to control for other confounding variables such as smoking status, antipsychotic drug treatment, and disease duration. Further improvements in clinical data collection as well as consensus on a set of guidelines to increase accessibility to meta-data will allow assessment of a greater number of confounding variables in future studies. This would thereby enhance statistical power and facilitate the progress of biomarker discovery.

Despite growing interest in exploring peripheral tissues for a better understanding of molecular mechanisms underlying psychiatric disorders, only a few genome-wide analyses have been conducted using blood tissue. As such, access to blood studies was limited compared to brain studies. Nevertheless, because of the simplicity of access to blood tissues, some investigators were able to recruit large cohorts of both controls and patients (de Jong et al., 2012; Gardiner et al., 2013). With inclusion of these two studies, we were able to have a balanced number of samples from our blood meta-analysis compared to our brain meta-

analysis. As we observed for the brain meta-analysis, dysregulated gene expression was well balanced between down- and up-regulation in blood samples from patients. When setting a statistical threshold ($q < 0.010$) that allows a similar number of top hits (slightly over 250 hits) compared to the brain meta-analysis, we observed that the protein kinase cascade and the inflammatory response/lymphocyte activation/cytokine binding were the only overrepresented biological processes that characterize SCZ. This finding is in agreement with the results highlighted by the individual studies our meta-analysis was built on (de Jong et al., 2012; Gardiner et al., 2013; Kumarasinghe et al., 2013; van Beveren et al., 2012), as well as other independent studies (Drexhage et al., 2010; Sainz et al., 2013; Sanders et al., 2013; Xu et al., 2012). In accordance with our brain meta-analysis, age and gender were included as covariates in the ANOVA on the blood cohort since they were significantly different between controls and patients (Table 2). Unfortunately, the clinical data available for the study was incomplete for smoking status, disease duration, the medication used, and whether the SCZ patient was in an acute phase or had been stabilized. As such, our analysis was supported by an increased study population rather than integration of all confounding variables.

Despite the classical difficulties encountered by investigators when addressing the episodic nature of SCZ, the heterogeneity in clinical presentation and the medications used by study participants when building large cohorts, we believe that any common characteristics unifying such diverse brain and blood cohorts is of high interest to understand SCZ pathophysiology. Indeed, ontological annotations presented here have proven that brain and blood share in common the dysregulation of defense/wounding/inflammatory responses. This is supported by a meta-signature of 13 genes in our analysis that are dysregulated in the same direction in both brain and blood (Fig. 1). Significantly, ten of the common dysregulated genes play a role in processes related to inflammation and the immune response. For example, *ABCF1*, ATP-binding cassette, sub-family F (GCN20), member 1, is located within the gene-

dense MHC region essential to the immune system that also contains many polymorphisms that have been implicated in SCZ (Ripke et al., 2013). *ABCF1* has been shown to be up-regulated in the blood of SCZ patients (Gardiner et al., 2013). It is also involved in the most highly connected intramodular hub in a co-expression module associated with SCZ that was independent of antipsychotic medication, as shown by its down-regulation in antipsychotic-free patient samples (de Jong et al., 2012).

ADM, encoding the hypotensive peptide adrenomedullin, was previously shown to be up-regulated in lymphoblastoid cells of patients in monozygotic twins discordant for SCZ (Kakiuchi et al., 2008). *BCL6*, B-cell lymphoma 6 protein, encodes a master transcription factor leading to the differentiation of naïve helper T cells (Nurieva et al., 2009). This gene has also been reported to control neurogenesis (Tiberi et al., 2012). Interestingly, BCL6 binds to the promoter of RGS4 to repress its transcription (Yang et al., 2010). *RGS4* is a candidate vulnerability gene that was found to be down-regulated in previous studies in SCZ (Bowden et al., 2007; Erdely et al., 2006; Levitt et al., 2006; Mirnics et al., 2001). *LCN2*, encoding lipocalin-2, also known as neutrophil gelatinase-associated lipocalin, is involved in limiting bacterial growth in innate immunity by iron sequestration (Yang et al., 2002). In another link with SCZ for *LCN2*, it was discovered that in the SCZ mice model *Disc1*-L100p, elevated levels of *Lcn2* transcripts due to *Disc1* mutation can be corrected by valproate. Furthermore, genetic deletion of Lcn2 normalized glial cell numbers and behavior in *Disc1*-L100P mutants (Lipina et al., 2012). *NCF4*, neutrophil cytosol factor 4, encodes a partner protein in the nicotinamide dinucleotide phosphate oxidase complex, a multi-component enzyme system whose main function is the elimination of invading microorganisms (Zhan et al., 1996). *S100A8*, encoding a calcium binding protein A8, calgranulin A, was first identified as up-regulated in the prefrontal cortex in SCZ by Maycox et al. (Maycox et al., 2009). A subsequent meta-analysis confirmed that the *S100A8* gene exhibited the greatest difference

between SCZ and control groups that was validated by RT-qPCR in an independent cohort (Perez-Santiago et al., 2012). Importantly, up-regulated transcript expression of *S100A8* in SCZ patients was also observed recently by RNA-Seq studies in blood samples (Xu et al., 2012). *S100A12*, encoding the pro-inflammatory S100 calcium binding protein A12, clagranulin C, had been previously reported to be upregulated in PBMCs from SCZ patients in three studies (Gardiner et al., 2013; Glatt et al., 2005; Middleton et al., 2005). *TSPO*, located near *NCF4* on the SCZ susceptibility chromosomal region 22q13.1, encodes a ubiquitous transmembrane protein localized to the outer mitochondrial membrane where it appears to form a multi-unit complex with voltage-dependent anion channels and adenine nucleotide translocators (Rupprecht et al., 2010). The protein has also been characterized as a peripheral benzodiazepine receptor (Papadopoulos et al., 2006), and acts as a molecular sensor for brain injury, mediating signaling between microglia and neurons to control the inflammatory response required for injury repair in the brain. Therefore, TSPO binding is considered a biomarker for neuroinflammation (Chen and Guilarte, 2008), and *TSPO* gene variants are candidates in the investigation of antipsychotic-induced weight gain (Pouget et al., 2015). *TLR2*, encoding the Toll-like receptor 2, plays an important role in innate immunity by sensing a variety of pathogens and is important in microglial activation. Since an exaggerated release of IL-1β, IL-6, and TNF-α has been detected after treatment with the TLR2 agonist, it has been speculated that *TLR2* may be involved in the pathogenesis of SCZ (Kang et al., 2013; McKernan et al., 2011).

Finally, we focused on another gene, *CX3CR1* (chemokine [C-X3-C Motif] receptor), which encodes the fractalkine (CX3CL1) receptor. In the brain, CX3CR1 is localized to microglia and CX3CL1 to neurons. Previous studies on CX3CL1/CX3CR1 signaling have provided insights into microglial-neuronal interactions in a variety of pathological conditions (Limatola and Ransohoff, 2014). Apart from a gene expression profile analysis of the locus

coeruleus of a patient with idiopathic Parkinson's disease (Cui et al., 2015), and an RNA screen in combination with flow cytometric analyses demonstrating a significantly lower expression of CX3CR1 in peripheral mononuclear cells in multiple sclerosis patients compared to healthy individuals (Infante-Duarte et al., 2005), the *CX3CR1* gene has not been previously mentioned as dysregulated in neuropsychiatry. We thus concluded that our results point to a possible role for *CX3CR1* in psychiatric disorders and used RT-qPCR to investigate its expression in PBMCs from an independent validation cohort of stabilized SCZ patients and healthy controls.

Importantly, we confirmed our meta-analysis data and observed a significant down-regulation of *CX3CR1* expression in SCZ patients compared to healthy controls. In this validation cohort we showed that this dysregulation of *CX3CR1* expression was independent of confounding variables, including tobacco smoking, which we had not been able to verify in our meta-analysis. Since we had more extensive clinical information related to the validation cohort, we assessed possible parameters that may be linked to dysregulated *CX3CR1* gene expression. Of all the parameters tested, the depression-anxiety phenotype was closely associated with chemokine receptor expression. Specifically, *CX3CR1* expression was correlated to a depression-anxiety dimension of 5-dimension PANSS with CDSS scores, and both subscales of the STAI. It is important to keep in mind that our validation cohort of patients was stabilized on treatment, therefore we should not exclude that the other more characteristic, acute clinical dimensions of SCZ (i.e. positive, negative and disorganisation symptoms) could have also been correlated to *CX3CR1* mRNA expression. Our observations lead us to explore the literature to determine what might be the link between a chemokine receptor and SCZ.

CX3CR1 is expressed by subsets of monocytes, dendritic cells, activated lymphocytes, and natural killer cells throughout the body. Since these cells rarely infiltrate the brain

parenchyma during normal physiological conditions, resident microglia are considered the only source of CX3CR1 expression, and thus the only recipient of fractalkine signaling (CX3CL1) in the healthy brain (Schulz et al., 2012). CX3CR1 is expressed in microglia throughout embryogenesis and during the murine lifespan (Ginhoux et al., 2010; Schulz et al., 2012). With accumulating evidence for activation of microglia and circulating monocytes in patients with bipolar disorder, major depressive disorder, and SCZ, investigators have been interested in tracing these cells in different animal models (Beumer et al., 2012). In particular, the developmental and neuroimmune roles of microglia have been addressed by the establishment and study of *Cx3cr1*-/- deficient mice. Corona et al. (2010) were the first to show that these mice exhibit prolonged social withdrawal and depression-like behavior after LPS injection (Corona et al., 2010). In fact, mice lacking *Cx3cr1* show a variety of neuronal defects thought to be the result of deficient microglia function. Activation of CX3CR1 is important for the proper migration of microglia to sites of injury and into the brain during development (Pagani et al., 2015). Moreover, mice lacking the CX3CR1 receptor show contextual fear conditioning, Morris water maze and motor learning deficits with a significant impairment in long-term potentiation (LTP) (Rogers et al., 2011). It has been recently shown that mice deficient for *Cx3cr1* exhibit a transient reduction of microglia during the early postnatal period and a consequent deficit in synaptic pruning that is associated with weak synaptic transmission, decreased functional brain connectivity, deficits in social interaction, and increased repetitive-behavior phenotypes (Zhan et al., 2014).

This meta-analysis identified 13 gene transcripts that are differentially expressed in the same direction in the blood and the brain from individuals with SCZ compared to healthy controls. We identified more genes (N=24, Fig. 1 and Supplementary Tables) that were dysregulated in both tissues, although with opposite directions of expression. Since no specific biological process was overrepresented with these 24 genes, and only two genes,

namely *MACF1* (Costas et al., 2013) and *TAP1* (Fellerhoff and Wank, 2009), had already been associated with SCZ, we believe that our search for a converging meta-signature provided the most relevant strategy to identify SCZ markers. In particular, this approach pinpointed CX3CR1 as a potential relevant marker involved in SCZ pathophysiology, particularly with regards to the neurodevelopmental theory of SCZ and potential roles for monocytes and microglia. Although further work is required to determine which specific cells in the peripheral blood have altered CX3CR1 expression, our results could be of great interest for monitoring clinical evaluation of a very complex disorder.

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Figure Legend

Fig. 1. Venn diagram depicting the distribution of dysregulated genes (underexpressed and overexpressed) in SCZ patients compared to control subjects from the brain and blood meta-analyses, using 0.001 and 0.05 as q-value thresholds respectively. The 13 genes that exhibit a similar pattern of dysregulation in both meta-analyses are indicated and were uploaded on the DAVID ontological tool to identify any over-represented biological process. The 10 genes in bold are the ones related to the wounding/defense/inflammatory response.

Table 1

Microarray data used in this study.

Database	Id	Reference	Platform (probe number)	Cohort	Tissue	Parameters considered for ANOVA
Stanley	6	Feinberg	Affymetrix, U95Av2 (12453)	28 CTL 22 SCZ	Cerebellum	
Stanley	14	Sklar	Affymetrix, U95Av2 (12453)	12 CTL 13 SCZ	Frontal Cortex (BA8-9)	
Stanley	18	Aston et al., 2005 (Aston et al., 2005)	Affymetrix, U95Av2 (12453)	19 CTL 15 SCZ	Temporal Cortex (BA21)	
Stanley	2	Altar	Affymetrix, HGU 133a (22283)	29 CTL 21 SCZ	Frontal Cortex (BA46-10)	
Stanley	16	Chu et al., 2009 (Chu et al., 2009)	Affymetrix, U133Plus2.0 (54681)	12 CTL 14 SCZ	Thalamus	
Stanley	17	Laeng	Affymetrix, U133Plus2.0 (54681)	21 CTL 20 SCZ	Hippocampus (CA1)	Cohort group, Experiment, Brain area, Age, Sex, PMI, pH
GEO, ArrayExpress	GSE17612	Maycox et al., 2009 (Maycox et al., 2009)	Affymetrix, U133Plus2.0 (54681)	23 CTL 28 SCZ	Prefrontal Cortex (BA10)	
GEO, ArrayExpress	GSE21138	Narayan et al., 2008 (Narayan et al., 2008)	Affymetrix, U133Plus2.0 (54681)	30 CTL 29 SCZ	Prefrontal Cortex (BA46)	
GEO, ArrayExpress	GSE21935	Barnes et al., 2011 (Barnes et al., 2011)	Affymetrix, U133Plus2.0 (54681)	19 CTL 23 SCZ	Temporal Cortex (BA22)	
GEO, ArrayExpress	GSE53987		Affymetrix, U133 Plus2.0 (54681)	19 CTL 19 SCZ	Hippocampus, Prefrontal Cortex (BA46) and Striatum	
GEO	GSE27383	van Beveren et al., 2012 (van Beveren et al., 2012)	Affymetrix, U133 Plus2.0 (54681)	29 CTL 43 SCZ	PBMCs	
GEO	GSE38481	de Jong et al., 2012 (de Jong et al., 2012)	Illumina HumanRef-8 v3.0 expression beadchip (24526)	22 CTL 15 SCZ	Whole Blood	
GEO	GSE38484	de Jong et al., 2012 (de Jong et al., 2012)	Illumina HumanHT-12 V3 expression beadchip (48803)	96 CTL 106 SCZ	Whole Blood	Cohort group, Experiment, Cell type, Age, Sex
		Gardiner et al., 2012 (Gardiner et al., 2013)	Illumina HumanHT-12 V3 expression beadchip (48803)	80 CTL 66 SCZ	PBMCs	
		Kumarasinghe et al., 2013 (Kumarasinghe et al., 2013)	Illumina HumanHT-12 V3 expression beadchip (48803)	11 CTL 10 SCZ	PBMCs	

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Table 2

Demographic variables across combined cohorts.

	Brain			Blood		
Group	Control	SCZ	p-value	Control	SCZ	p-value
Age (years)	50.5 ± 15.5	50.2 ± 17.8	0.864[a]	36.2 ± 14.2	36.3 ± 12.1	0.914
Gender (F/M)	91/156	81/153	0.255[b]	112/126	63/177	**3.7E-13**[b]
pH	6.46 ± 0.31	6.27 ± 0.30	**0.013**[a]			
PMI	22.5 ± 12.5	25.8 ± 16.2	**2.2E-7**[a]			

Results are shown as mean ± SD.

[a] Student's t-test

[b] χ^2 test

Table 3

Gene ontology analysis of dysregulated genes in brain meta-analysis (q ≤ 0.0001).

ID	Term	Genes	Count	%	p-value	FDR
GO:0010033	Response to organic substance	**ADM**, ATP5G3, CALCOCO2, CDKN1A, CLIC1, EPHX1, GOT1, GSTM3, HSPB1, IL1R1, IRS2, MSH2, NFKBIA, PLIN2, PPP3CB, PRKACB, RNF14, SERPINH1, SH2B2, SHC1, SLC11A1, SOCS3, SST, STAT3, TAC1, TAF9, TNFRSF1A, TOR1A, TXNIP, UQCRFS1	30	11.3	1.1E-5	0.02
GO:0048584	Positive regulation of response to stimulus	BECN1, C1R, EEF1E1, IRF7, NFKBIA, POLR3C, POLR3F, SERPING1, SH2B2, SLC11A1, TAC1, TAF9, TGM2, TNFRSF1A, UBE2N	15	5.7	4.5E-5	0.08
GO:0033554	Cellular response to stress	**ADM**, AIFM1, BCL6, BECN1, CDKN1A, CHST3, CUL4B, FEN1, GTF2H1, INSIG1, IRF7, MSH2, MT3, NAE1, NUPR1, PXN, RAD17, RAD51C, RBM38, RPA1, RPA3, SUMO1, UBE2N, WFS1	24	9.1	8.1E-5	0.14
GO:0002684	Positive regulation of immune system process	BCL6, C1R, CDKN1A, IL4R, NFKBIA, POLR3C, POLR3F, SERPING1, SH2B2, SLC11A1, SOCS5, TAC1, UBE2N	13	4.9	6.9E-4	1.17
GO:0006954	**Inflammatory response**	ATRN, CEBPB, C1R, IRF7, MAP2K3, NUPR1, **S100A8**, S100A9, SERPINA3, SERPING1, SLC11A1, STAT3, TAC1, TNFRSF1A	14	5.3	3.4E-3	5.55
GO:0009611	**Response to wounding**	ACVRL1, **ADM**, ATRN, C1R, CEBPB, CHST3, **CX3CR1**, IRF7, MAP2K3, NUPR1, PIK3CB, **S100A8**, S100A9, SERPINA3, SERPING1, SLC11A1, STAT3, TAC1, TNFRSF1A	19	7.2	3.6E-3	5.88
GO:0006952	**Defense response**	ATRN, BECN1, C1R, CALCOCO2, CEBPB, CLIC1, **CX3CR1**, FGR, IL1R1, INHBB, IRF7, MAP2K3, NUPR1, **S100A8**, S100A9, SERPINA3, SERPING1, SLC11A1, STAT3, TAC1, TNFRSF1A	21	7.9	3.6E-3	5.92
GO:0000502	Proteasome complex	HSPB1, PSMA2, PSMA5, PSMB3, PSMB6, PSMC6, PSMD10, PSME3	8	3.0	5.2E-5	0.07
GO:0051443	Positive regulation of ubiquitin-protein ligase activity	PSMA2, PSMA5, PSMB3, PSMB6, PSMC6, PSMD10, PSME3, UBE2N	8	3.0	1.7E-4	0.28
GO:0032268	Regulation of cellular protein metabolic process	CCND3, DDX1, EIF4E, EIF5, HSPB1, METAP1, MKNK2, NFKBIA, PSMA2, PSMA5, PSMB3, PSMB6, PSMC6, PSMD10, PSME3, SERPING1, SOCS3, TAF9, TIMP1, UBE2N	20	7.6	4.1E-4	0.69
GO:0009057	Macromolecule catabolic process	AIFM1, AUH, CAND1, CHI3L1, CUL4B, FEN1, GTF2H1, HYAL2, NAE1, PSMA2, PSMA5, PSMB3, PSMB6, PSMC6, PSMD10, PSME3, RNF14, RPA1, RPA3, SOCS3, SOCS5, SUMO1, UBE2D2, UBE2E3, UBE2N, UBE2S, ZBTB16	27	10,2	6.5E-4	1.10
GO:006091	Generation of precursor metabolites and energy	ATP5G3, ATP6V0D1, DLAT, DLD, FADS3, GOT1, GYS1, IDH3A, MSH2, NDUFA9, NDUFS2, PDHB, SLC25A12, UQCRFS1, UQCRC2	15	5.7	8.3E-4	1.40
GO:0005739	Mitochondrion	AIFM1, ALDH4A1, ATP5G3, AUH, BCAT2, C10orf10, DLAT, DLD, FEN1, HCCS, IDH3A, ISCA1, LIMK2, LRPPRC, MLXIP, NDUFA9, NDUFS2, PDHB, PPP3CB, PTS, SHC1, SLC25A5, SLC25A12, SLC25A46, STOML2, TGM2, TIMM17A, TXNIP, UQCRC2, UQCRFS1	30	11.3	4.5E-3	5.89
GO:0005507	Copper ion binding	CP, MT1E, MT1F, MT1G, MT1H, MT1X, MT3	7	2.6	9.4E-4	1.36

Biological processes and genes also observed for blood meta-analysis are indicated in bold.

Table 4

Gene ontology analysis of dysregulated genes in blood meta-analysis (q ≤ 0.01).

ID	Term	Genes	Count	%	p-value	FDR
GO:0006952	**Defense response**	ABCF1, CAMP, CCL5, CD302, CD40LG, CD74, **CX3CR1**, DEFA4, HP, IL27RA, ITGAL, LY96, NCR3, NFATC3, PRF1, PROK2, **S100A8**, SIGIRR, THBS1, TLR2, TLR10	21	8.2	5.8E-4	0.96
GO:0009611	**Response to wounding**	ABCF1, **ADM**, CCL5, CD302, CD40LG, **CX3CR1**, ENTPD1, F5, ITGAL, JAK2, LY96, NCR3, NFATC3, PROK2, **S100A8**, SIGIRR, THBS1, TLR2, TLR10	19	7.5	6.6E-4	1.10
GO:0006954	**Inflammatory response**	ABCF1, CCL5, CD302, CD40LG, ITGAL, LY96, NCR3, NFATC3, PROK2, **S100A8**, SIGIRR, THBS1, TLR2, TLR10	14	5.5	8.7E-4	1.44
GO:0045321	Leukocyte activation	ADA, CARD11, CBLB, CD7, CD8A, CD40LG, CD74, EOMES, FYN, GAPT, ITGAL, SKAP2, TLR2	13	5.1	2.0E-4	0.34
GO:0019955	Cytokine binding	**CX3CR1**, CXCR3, CD74, IL27RA, IL2RB, IL10RA, THBS1	7	2.8	4.2E-3	5.74
GO:0007243	Protein kinase cascade	AKAP11, CD74, CD81, FYN, IRF3, JAK2, LRRN3, LY96, MAP4K1, PPP2R1A, PROK2, THBS1, TLR2, TLR10	14	5.5	2.8E-3	4.54

Biological processes and genes also observed for brain meta-analysis are indicated in bold.

Table 5

Validation cohort characteristics.

		SCZ (n=29)	Control (n=31)	Statistics
Age (years)		34.3 ± 9.4	32.7 ± 7.4	p = 0.464[a]
Gender (F/M)		12/17	12/19	p = 0.833[b]
Tobacco smoking (yes/no)		20/9	13/18	p = **0.035**[b]
Duration of illness (years)		10.9 ± 7.3		
Antipsychotics used				
	risperdone	13		
	aripiprazole	16		
Chlorpromazine equivalent dose (mg/d)		311.9 ± 240.1		
PANSS classical 3-dimension scores				
	total score	47.2 ± 17.7		
	positive subscale	7.2 ± 5.9		
	negative subscale	15.4 ± 7.3		
	general psychopathology	24.6 ± 8.9		
PANSS 5-factor scores				
	negative	17.3 ± 9.5		
	positive	8.3 ± 5.2		
	disorganization	12.3 ± 4.9		
	psychomotor excitation	2.7 ± 3.3		
	depression/anxiety	6.5 ± 4.0		
CDSS		2.1 ± 1.6		
S-Anxiety		39.5 ± 10.5		
T-Anxiety		45.2 ± 8.9		

PANSS: Positive and Negative Syndrome Scale.

CDSS: Calgary Depression Scale for Schizophrenia.

S-Anxiety: State subscale of the State-Trait Anxiety Inventory.

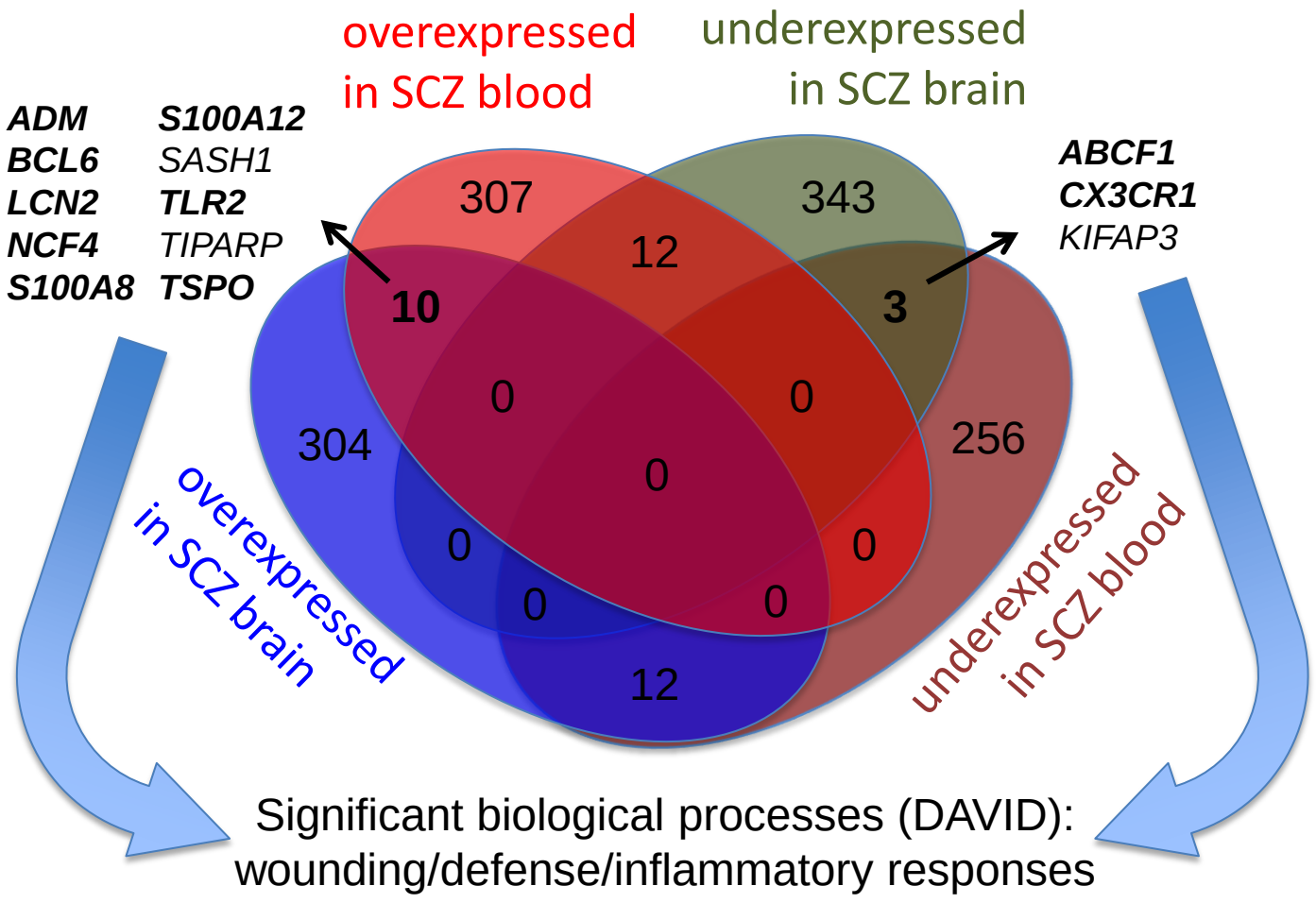
T-Anxiety: Trait subscale of the State-Trait Anxiety Inventory.

Results are shown as mean ± SD.

[a] Student's t-test.

[b] χ^2 test.

Figure(s)


overexpressed
in SCZ blood
underexpressed
in SCZ brain
ADM
BCL6
LCN2
NCF4
S100A8
S100A12
SASH1
TLR2
TIPARP
TSPO
ABCF1
CX3CR1
KIFAP3
307
343
12
10
3
0
0
304
0
256
0
0
0
0
12
overexpressed
in SCZ brain
underexpressed
in SCZ blood
Significant biological processes (DAVID):
wounding/defense/inflammatory responses

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Conflict of interest

The authors have no conflicts of interest to disclose.

Contributors

R. Belzeaux, E. Fakra and E. C. Ibrahim designed the study. A. Bergon performed the bioinformatics work. M. Comte and E. Fakra recruited study subjects and provided the clinical data concerning the validation cohort. F. Pelletier, M. Hervé and E. C. Ibrahim performed the experiments. A. Bergon, R. Belzeaux and E. C. Ibrahim undertook the statistical analysis. E. J. Gardiner, N. J. Beveridge, B. Lui, V. Carr, R. J. Scott, B. Kelly, M. J. Cairns, Nishantha Kumarasinghe, Ulrich Schall, and P. A. Tooney provided raw data from blood samples of SCZ and controls that were not on public repository. R. Belzeaux, O. Blin, J. Boucraut, E. Fakra and E. C. Ibrahim obtained funding for the study. E. C. Ibrahim conducted the literature search and wrote the first draft of the manuscript. R. Belzeaux, E. Fakra, P. A. Tooney and E.C. Ibrahim contributed to write the final manuscript. All authors have approved the final manuscript.

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