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Blood plasma proteomic modulation induced by olanzapine and risperidone in schizophrenia patients

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ABSTRACT

Antipsychotics are the main line of treatment for schizophrenia. Even though there are significant rates of medication drop out due to side effects and limited response of approximately 50% of patients. This is likely due to incomplete knowledge in how these drugs act at the molecular level. To improve treatment efficacy during the critical early stages of schizophrenia, we aimed to identify molecular signatures at baseline (T0) for prediction of a positive response to the atypical antipsychotics olanzapine and risperidone after 6 weeks (T6) treatment. Blood plasma samples were processed and analyzed by label-free quantitative shotgun proteomics using two-dimensional nano-liquid chromatography, coupled online to a Synapt G2-Si mass spectrometer. Data were obtained in MS^E mode (data-independent acquisition) in combination with ion-mobility ($HDMS^E$). We were able to identify a potential panel of proteins that might predict a positive outcome to olanzapine and risperidone treatment. The proteins found to be differentially abundant between T0 and T6 in good responders compared to poor responders were analyzed in silico for enrichment pathways and found to be mostly involved with immune system functions. This data can contribute to better understand the biochemical signaling mechanisms peripherally triggered by antipsychotic medication and eventually used to develop surrogate biomarker tests to help improve treatment outcomes and guide development of new treatment approaches.

Significance: The application of proteomics to the study of the atypical antipsychotic effects on the blood plasma proteome from schizophrenia patients could help in the search for new targets to improve the current therapies, as well as in the development of new therapeutic strategies. In this original article, we provided clues that atypical antipsychotics might be associated with good response by modulating proteins that play a role in inflammation and/or immune system pathways. In addition, the proteins with differential abundance found in the comparison between good and poor responders at the baseline might compose a signature for prediction of response effectiveness.

1. Introduction

The combination of environmental and genetic factors leads to chronic disability such as hallucinations, delusions and cognitive symptoms commonly observed in schizophrenia, one of the most grievous mental disorders affecting around 21 million people globally [1–3].

Antipsychotics show limited response [> 20% Positive and Negative Syndrome Scale (PANSS) / Brief Psychiatric Rating Scale (BPRS) reduction or a Clinical Global Impression (CGI) score indicating "slightly improved") in approximately 50% of patients and side effects often cause medication drop out [4]. Nonetheless, they have been the main line in the therapeutics of schizophrenia because of their capacity to reduce psychotic symptoms and prevent relapse [4,5]. However, the

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Table 1
Patient demographics and baseline characteristics.

	Risperidone GR	Risperidone PR	Olanzapine GR	Olanzapine PR
N	8	7	6	5
PANSS total score (T6) mean ± SD	34.25 ± 12.26	20.85 ± 7.26	43.00 ± 8.46	22.60 ± 23.75
Symptom reduction from T0 to T6 [% change in PANSS total, mean ± SD]	0.69 ± 0.16	0.36 ± 0.05	0.75 ± 0.11	0.30 ± 0.25
Age, years, mean	39.88	40.00	34.83	31.20
Age interval	30–58	16–56	25–50	23–50
Illness duration (Years)[#]	8.7 ± 7.73	16.06 ± 12.6	13.07 ± 12.2	7.4 ± 8.34
Sex (male), n(%)	87.50	57.14	50.00	60.00
smokers (%)	42.85	57.14	83.33	60.00
BMI (kg/m^2) mean ± SD	23.20 ± 3.98	23.80 ± 3.60	26.70 ± 3.87	24.80 ± 2.86

Abbreviations: N, total number of patients; n, number of affected patients; corrected PANSS, Positive and Negative Syndrome Scale scores are displayed (subtraction of minimum scores representing "no symptoms" from the PANSS scores); BMI, body mass index; SD, standard deviation. Positive clinical response ⩾ 50% of total reduction from PANSS, corrected. [#]Refers to patients who were acutely ill and unmedicated but did not belong to the drug-naive first-episode group.

most troubling fact is that patients without adequate treatment cannot function in society. This can lead to high expenses related to hospitalization and treatment, as well as a continual reduction in quality of life for the patients [6]. Antipsychotic medications target mainly dopamine receptors with different levels of affinity, and early intervention has been shown to improve efficacy and the long-term course for patients [7].

Atypical antipsychotics, such as risperidone and olanzapine, were approved for treatment of schizophrenia by the Food and Drug Administration in the 1990s. In addition to their role as antagonists of dopamine D_2 receptors, they act on a wide range of receptors, conferring both therapeutic and side effects, with the latter mostly related to metabolic syndrome [8]. Olanzapine is considered to be one of the most effective medications in the treatment of chronic schizophrenia [9]. Also, olanzapine treatment generally leads to improvements in PANSS scores, as well as reductions in the rates of hospitalization and drop outs [10,11]. However, it can also induce side effects associated with weight gain, dyslipidemia, and elevated levels of transaminases. Risperidone is an effective treatment option for both newly diagnosed and chronic schizophrenia patients. Although considered atypical, risperidone may also present a typical antipsychotic profile, depending on the dosage used, by causing extrapyramidal side-effects and an elevation in prolactin levels. In addition, risperidone has been documented to cause weight gain and sedation to a lesser extent than olanzapine [12,13].

Due to the prevalence of side effects caused by antipsychotic treatment, including metabolic syndrome (average 36%), weight gain and obesity (average 30%), and cardiovascular effects (16–49%), and compounded with the medium efficacy of antipsychotics, approximately 50% of patients eventually abandon treatment [14–16]. As such, biomarkers that can be used to indicate efficacy, or lack thereof, for a given drug must be identified.

In all higher organisms, the homeostatic mechanisms of the brain work in concert with the periphery and this is mediated by a number of two-way communication networks, such as the bloodstream. In the early 1960s, Frank Putnam [17] was a pioneer for studies of whole-blood, plasma proteins. Almost 60 years later, the search for the best representation of the human blood plasma/serum proteome is still a moving target, propelled by the rapid advancements of proteomic techniques and instrumentation. The interest in the plasma/serum proteome is multifaceted. The ease of access makes this body fluid attractive for medical applications and collecting blood is one of the least invasive techniques. It is routine not only in hospitals, but also in clinical and research laboratories worldwide. Clinical applications are numerous, with disease diagnosis and drug reaction monitoring being some of the most valued, such as those affecting the brain-periphery axes [18–20]. Therefore, analysis of blood plasma molecules can be a useful approach to show disturbances in the brain and/or the effects of a treatment. By definition, proteomics is a suitable tool for studies of complex diseases such as brain disorders, because of its ability to identify, quantify, and characterize numerous proteins across different conditions [21].

Proteomics emerged in the post-genomic era as a strategy for the identification of proteins and biochemical pathways involved in the pathophysiology of human diseases. Consequently, discovering proteins which present differential abundances under specific conditions may represent potential biomarkers. These conditions can include both physical conditions, such as diseases and stress, as well as chemical conditions, including drug treatments. In clinical psychiatry, biomarkers have been researched in body fluids since the 1910s [22]. However, discovering proteomic blood markers is one of the most challenging tasks in analytical terms, since at least ten orders of magnitude separate the least abundant from the most abundant proteins [23]. Thus, techniques to deplete abundant proteins were developed which allowed more precise studies on biomarkers discovery [24]. Protein signatures have been sought in order to predict a positive response to psychiatric treatment, but there is still lack of relevant molecules to compose a panel of proteins for a positive outcome to specific antipsychotics [25–28].

The blood plasma/serum proteome has been investigated for identification of potential biomarkers that may improve clinical outcomes by being reliable predictors of the right medication in the early course of schizophrenia [29]. With this in mind, we aimed to define molecular profiles in the plasma of drug naïve/drug free schizophrenia patients, before treatment with olanzapine and risperidone. Also, we analyzed blood plasma proteins from SCZ patients treated with an atypical antipsychotic to better understand the pathways involved in response effectiveness.

2. Material and methods

2.1. Blood samples

Blood plasma samples (Table 1, Supplementary Table 1) were collected at 08:00 am by venous puncture from acutely ill SCZ subjects (n = 26) who were un-medicated for at least 6 weeks prior to inclusion [designated time zero (T0)] and again after 6 weeks (T6) of treatment with the antipsychotics olanzapine (n = 11) and risperidone (n = 15). After the 6-week treatment period, all samples were grouped according to those patients who responded (olanzapine n = 6, risperidone n = 8) or did not respond (olanzapine n = 5, risperidone n = 7) favorably to the treatment. A favorable response was defined as a 50% reduction in total PANSS scores. These scores were corrected by subtraction of the minimum scores which represented no symptoms (7, 7, and 16 for PANSS positive, negative and general scores, respectively) [30]. During the treatment period patients were hospitalized. Medical conditions such as substance abuse disorder or symptoms induced by a non-psychiatric medical illness or treatment, including immune diseases, immunomodulatory treatment, cancer, chronic terminal disease, cardiovascular disorders, dyslipidemia, diabetes, and severe trauma were

causes for exclusion from the study. The ethics committee of the Psychiatric Clinic of the University of Magdeburg, Germany, approved the procedures for sample collection and analysis (process 110/07, from November 26th, 2007 amended on February 11th, 2013). The purpose of the study was also explained to subjects and informed, written consent was obtained. After collection of blood at T0 and T6 into plasma tubes, the samples were immediately centrifuged for 10 min at 2000 ×*g*. The plasma supernatants were aliquoted and stored at −80 °C.

2.2. Sample preparation prior to LC-MS

To decrease the dynamic range for the analyses [31–33], 30 μL of blood plasma from each donor was immunodepleted of 14 high-abundance proteins (alpha-1-antitrypsin, A1-acid glycoprotein, serum albumin, alpha2-macroglobulin, apolipoprotein A-I, apolipoptrotein A-II, complement C3, fibrinogen alpha/beta/gamma, haptoglobin, IgG A, IgG G, IgG M, transthyretin, and serotransferrin) using the Hu14 Affinity Removal System (Agilent; Wokingham, UK) coupled to a high-performance liquid chromatography system (Waters HPLC system with 2487 Dual λ Absorbance Detector) (Supplementary fig. 1). The manufacturer's protocol was followed for the depletion procedure as detailed in Garcia and collaborators [34,35]. Briefly, 30 μL plasma was diluted to 120 μL in phosphate buffer and injected into a liquid chromatography system at a flow rate of 0.125 mL/min for 18 min. The unbound effluent (1.25 mL) containing enriched, low-abundance proteins were buffer-exchanged into 50 mM ammonium bicarbonate with Vivaspin 6 cartridges (Sartorius). The concentration of the proteins in the resulting 500uL fraction were determined using the Qubit® Protein Assay Kit with Qubit® 3.0 Fluorometer following the manufacturer's protocol (Thermo Scientific, Loughborough, UK). On average, we obtained 0.5 μg/μL of proteins in each sample. Proteins (50 μg) were reduced with dithiothreitol (100 mM, 30 min, 60 °C) and alkylated with iodoacetamide (300 mM, 30 min, room temperature, protected from light). Proteins were digested with trypsin (Promega, Heidelberg, Germany) at a ratio of 1:100 (*w*/w trypsin:protein) overnight at 37 °C prior to 2D-nanoUPLC-HDMS^E analysis as described previously [34]. This procedure was also performed for the bound fraction and the eluate containing the high abundance protein fraction was analyzed in a separate study [36]. The peptide concentration was determined as above.

2.3. 2D-UPLC-HDMSE analyses

Peptides were injected into a 2D-RP/RP nanoAcquity UPLC M-Class System (Waters Corporation, Milford, MA, USA), coupled online to a Synapt G2-*Si* mass spectrometer (Waters Corporation). Mobile phase A was formic acid (0.1%) in LCMS grade water and B was 0.1% formic acid in acetonitrile. To prevent bias during the measurements, data acquisition was randomized for each group. Also, the reference calibration was performed frequently between runs to avoid mass scale drift. In addition, runs using the trap and the analytical columns with one blank sample were done to avoid carryover between the samples.

Peptides (2.5 μg) were then loaded onto an M-Class BEH C18 Column (130 Å, 5 μm, 300 μm × 50 mm, Waters Corporation) for first-dimension chromatography, was performed through five discontinuous steps of mobile phase B (11%, 14%, 17%, 20%, and 50%) at high-pH fractionation for 10 min at a flow rate of 2 μL/min. After each step, peptide loads were subjected to second-dimension separation in a nanoACQUITY UPLC HSS T3 Column (1.8 μm, 75 μm × 150 mm, Waters Corporation, Milford, MA). Peptides trapped in the second dimension were eluted with 7% to 40% (*v*/v) mobile phase B over 29 min at a flow rate of 0.5 μL/min, directly into a Synapt G2-Si.

The mass spectrometer measured samples in MS^E mode, acquiring data while switching between low and high energy, with no selection window and a continuous ion current. The label-free quantification using MS^E mode, overcame some issues related to reproducibility, as both precursor and product ion information is collected on all isotopes of all charge-states of the eluting ions across the chromatographic peak width. This allows a sufficient number of points on the chromatographic peak to guarantee precise measurement of the *m/z*, retention time, and peak volumes of all detectable ions [37].The mass spectrometer calibration was performed with an MS/MS spectrum of human doubly charged [Glu1]-Fibrinopeptide B (785.8426 *m/z*) at 100 fmol/μL delivered every 30 s through the reference sprayer of the NanoLock Spray source. The injections were performed by nano-electrospray ionization in positive ion mode nanoESI (+) using a NanoLock Spray (Waters Corporation, Manchester, UK) ionization source for internal calibration.

2.4. Protein identification

Proteins were identified using dedicated algorithms and were searched against the UniProt reviewed human proteomic database (January 2019; 20,402 entries). For spectral processing and database searching, we used the Progenesis QI for Proteomics (QIP) software package with Apex3D, Peptide 3D, and Ion Accounting Informatics (Waters Corporation). Progenesis QIP starts with LC-MS data loading, and 150 counts marked the low-energy threshold, 50 counts were used for the elevated energy threshold, and 750 counts for the intensity threshold. Then, alignment and peak detection were performed, which creates a list of peptide ions of interest (hereafter referred to as peptides), explored within Peptide Ion Stats by multivariate statistical methods. The final step determines protein identity with the anion-accounting algorithm within Progenesis QIP [38]**.** The following parameters were considered to identify peptides: 1) digestion by trypsin with at most two missed cleavages; 2) variable oxidation (methioine) and fixed modification by carbamidomethyl (cysteine); 3) false discovery rate (FDR) less than 1% across all conditions (calculated using a reversed database created on-the-fly by Progenesis QIP; and 4) a mass error of less than 20 ppm. Identifications that did not satisfy these criteria were rejected. Also, as a default parameter, protein grouping was applied, which hides proteins containing peptides that are present in other proteins. Peptides associated with keratins were removed from our data.

2.5. Protein quantitation

The method applied for this study was relative quantification using non-conflicting peptides. The protein abundance in a run is performed using only peptides that are not part of another protein hit, applied as the sum of all unique normalized peptide ion abundances corresponding to that protein. Despite the possibility that this method may sacrifice data, it allows greater accuracy in quantification since it eliminates the effect of overlapping trends derived from conflicting peptides, associated with other proteins. The peptide ranking is done across all the runs. The abundances of the peptides are then averaged to provide an overall abundance for the protein. Details about data processing of the Progenesis software are described on the software website (www.nonlinear.com).

2.6. In silico analyses

To interpret the functional significance of the biological processes in differentially expressed proteins, we used DAVID (the Database for Annotation, Visualization and Integrated Discovery – https://david.ncifcrf.gov/) bioinformatics resources with the new GO category (GO Direct), considering a *p*-value less than 0.05 [39].

We evaluated the biological significance of the enriched proteins in pathway analysis using both, Ingenuity Pathways Knowledgebase (IPKB) through the algorithm Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Qiagen, Redwood, CA, United States) [40] to identify over-represented canonical pathways associated with the dataset,

and the Reactome Knowledgebase [41]. We used the IPA regulation *Z*-score algorithm to identify canonical pathways that are expected to be more active or less active. All default software parameters were used. The Reactome Knowledgebase (https://reactome.org) is a public database that allows network analysis with molecular details of signal transduction, transport, DNA replication, metabolism, and other cellular processes in a single consistent data model.

Spearman's rank-correlation coefficients with two tails were calculated to assess the associations of protein measures with the severity of clinical symptoms (PANSS). All statistical analyses were performed using the GraphPad Prism software (La Jolla, CA, USA). We applied a significance threshold of $p < .05$.

3. Results

3.1. Baseline analyses

The proteome profiling study to identify blood plasma protein signatures in drug naïve/drug free schizophrenia patients for response prediction to olanzapine and risperidone antipsychotics resulted in identification of 19,945 peptide ions in the olanzapine group and 14,197 peptide ions in the risperidone group, leading to identification of 10,972 and 8166 peptides, which correspond to 319 and 251 proteins, respectively.

The quantitative analysis was performed by comparison at baseline (T0) between good responders and poor responders for olanzapine and risperidone. Leading to identification of 14 proteins with altered levels in olanzapine, and 40 proteins with altered levels in risperidone, considering ANOVA < 0.1 (supplementary tables 2 and 3).

The correlation analysis between PANNS score and demographic data (BMI and age) was not statistically significant. Also, analysis of the 40 proteins detected in the risperidone baseline group resulted in negative correlations between PANNS score and normalized intensities of F5, PCLO, AMBP, LUM, CLU, DMXL2, PRPH2, ATRN, CFB, DEFA1, HSPA5, F10, SERPINC1, qsox1, AFM, GGH, KTN1-AS1, ITIH3, MBL2, ACTN2 and positive correlation of RBM44 (Table 2).

We plotted a volcano chart to highlight the most significant proteins in terms of relative abundance between good and poor responders. Also, up-regulated and down-regulated proteins are visualized on right and left side of the blue line, respectively (Fig. 1A, C). Also, we plotted a dynamic range chart of the identified proteins (Fig. 1B, D).

Label-free mass spectrometry profiling of baseline analyses (T0) revealed that the relative abundance of several proteins was different in good responders compared to those with a poor outcome. Proteins in our data are involved in 6 and 10 different biological processes in the olanzapine and risperidone groups, respectively (Fig. 2, supplementary table 2, 3).

Aiming to focus on potential protein signatures of treatment response, instead of broad molecular pathways, we performed hierarchical cluster analysis and heatmap using all differentially abundant proteins in olanzapine group ($n = 14$) and risperidone ($n = 40$), to show the expression changes between good and poor responder to these groups (Fig. 3A, B).

The comparison of the two signatures demonstrated a high-functional correlation of the protein profiles of both treatments, and protein metabolism and immune system were the major biological processes represented by both olanzapine and risperidone groups, respectively. Some of the proteins with significant differences between good and poor responders to olanzapine and risperidone in our data at baseline comparison have been previously implicated in schizophrenia. This include heterogeneous nuclear ribonucleoproteins A2/B1 [43–45], beta-Ala-His dipeptidase [46–49] insulin-like growth factor 2 [50–54], and piccolo protein [55–58].

Interestingly, a substantial portion of the proteins identified were unclassified in terms of biological processes (10% in olanzapine and 14% in risperidone), and some of them have a lack of experimental evidence at the protein level. These novel proteins may represent new targets in further studies related to biomarker discovery and disease mechanisms.

Table 2
The risperidone T0 group correlation between PANNS score and study dataset.

Variables T0 GRvsPR	*r*	Range	P-value
Age	0.13	−0.42 - 0.61	0.6381
BMI	0.05	−0.48 - 0.56	0.8496
F5	−0.75	−0.91 - -0.37	0.0013*
PCLO	−0.73	−0.91 - -0.34	0.0019*
AMBP	−0.70	−0.90 - -0.28	0.0037*
LUM	−0.66	−0.88 - -0.20	0.0078*
CLU	−0.66	−0.88 - -0.20	0.0078*
DMXL2	−0.64	−0.87 - -0.18	0.0097*
PRPH2	−0.64	−0.87 - -0.17	0.0103*
ATRN	−0.62	−0.86 - -0.14	0.0134*
CFB	−0.62	−0.86 - -0.14	0.0134*
DEFA1	−0.61	−0.86 - -0.13	0.0156*
HSPA5	−0.60	−0.85 - -0.11	0.0181*
F10	−0.59	−0.85 - -0.10	0.0198*
SERPINC1	−0.59	−0.85 - -0.09	0.0218*
qsox1	−0.57	−0.84 - -0.07	0.0261*
AFM	−0.57	−0.84 - -0.06	0.0272*
GGH	−0.56	−0.84 - -0.05	0.0297*
KTN1-AS1	0.55	0.04–0.83	0.0337*
ITIH3	0.54	0.01–0.83	0.0396*
MBL2	−0.53	−0.82 - -0.00	0.0445*
ACTN2	0.51	−0.01 - 0.82	0.0498*
C6	−0.50	−0.81 - 0.03	0.0557
POTEE	−0.44	−0.79 - 0.11	0.0983
RBM44	0.61	0.13–0.86	0.0148*
IGF2	0.48	−0.06 - 0.80	0.0689
CFD	0.50	−0.03 - 0.81	0.0577

BMI, body mass index; PANSS, positive and negative simptoms scale.
[a]Spearman's correlation coeficient was used for correlation analysis between PANSS and demographics data and proteins with differential abundance.
* *p value* lesser than 0.05.

3.2. Longitudinal analyses

After 6 weeks treatment of 26 schizophrenia patients with risperidone or olanzapine, 14 patients were classified as good responders according to the criteria outlined in the methods section (supplementary table 1). Of these 8 had been treated with risperidone (RGR) and 6 with olanzapine (OGR). Analysis of the blood plasma proteome using 2D-UPLC-HDMSE resulted in identification of 18,421 peptides ions in the risperidone group and 22,211 peptides ions in the olanzapine group leading to identification of 9802 and 11,919 peptides, which correspond to 376 and 382 proteins, respectively (Fig. 4). The quantitative analysis was performed by comparing treatment (T6) to baseline (T0) proteomes in both —GR and PR groups for olanzapine and risperidone.

Proteins differentially abundant between the aforementioned groups were analyzed for gene ontology related to biological processes and signaling pathways. For the GR groups (RGR and OGR), modulation of inflammatory proteins was the most over-represented function. For the PR groups, there were minimal overlaps with the GR group in the case of biological processes, and only one canonical pathway was affected but with weak statistical power (related to *Z*-score).

For proteins identified following the olanzapine treatment, we found 81 from the OGR group and 53 from the OPR to be differentially regulated (Fig. 5, Supplementary Tables 4, 5). These proteins were mostly involved with biological processes such as immune (OGR) and metabolic (OPR) pathways. In the case of risperidone, 94 proteins from RGR were differentially regulated and these were predominantly associated with the immune system and the 44 altered proteins in RPR group were linked to cellular function (Fig. 5, Supplementary Tables 6).

Additionally, the OGR group was found to contain an enrichment of pathways (Fig. 6, Table 3, Supplementary Tables 7,8) such as intrinsic

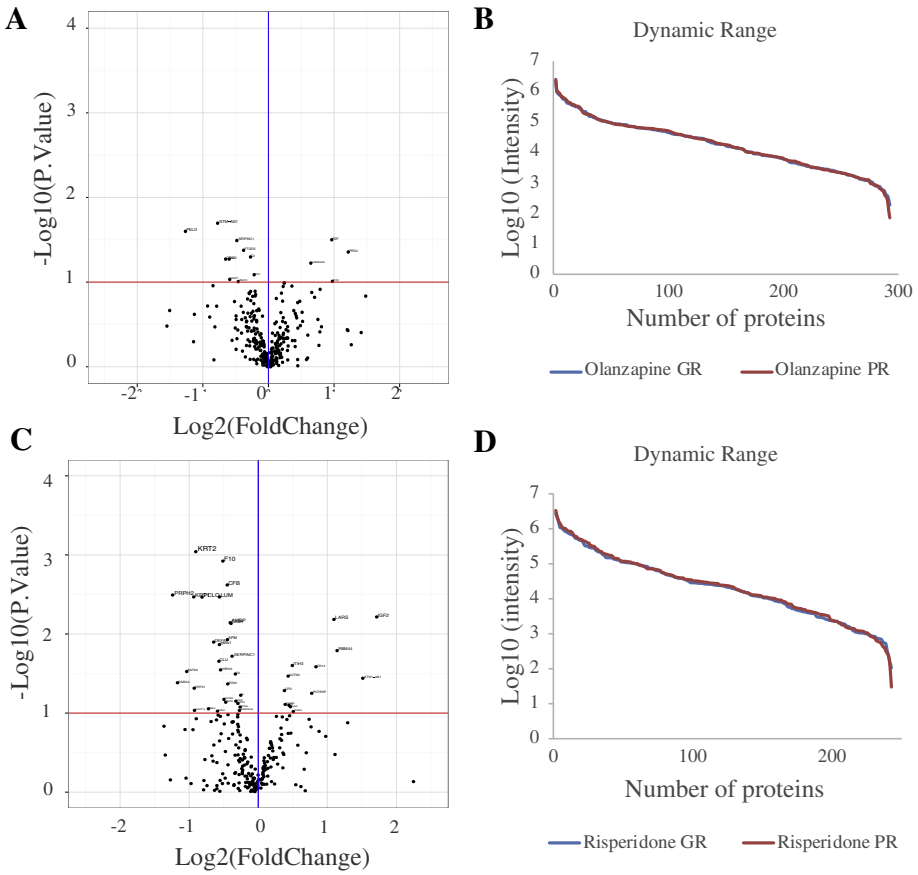

Fig. 1. Data analysis of the olanzapine and risperidone groups. (A, B) Volcano plot and dynamic range of quantified proteins from olanzapine and (C, D) risperidone groups.

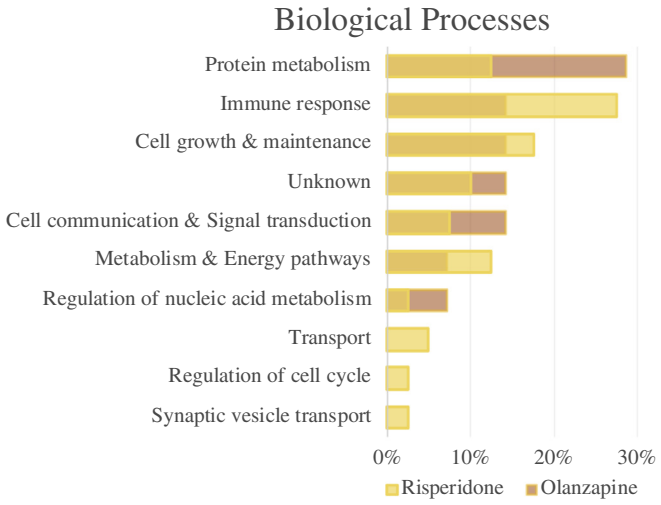

Fig. 2. Biological processes (HPRD) comparing olanzapine and risperidone.

prothrombin activation, coagulation system/clotting cascade, acute phase response signaling, production of nitric oxide and reactive oxygen species in macrophages and complement system, decrease of activation of LXR/RXR activation in OGR and OPR. RGR data were functionally annotated for pathways (Fig. 6, Table 3, Supplementary Tables 7, 8) and were mostly involved with LXR/RXR activation, decrease of activation of acute phase response signaling, production of nitric oxide and reactive oxygen species in macrophages, innate immune system, neutrophil degranulation and complement system.

3.3. Common canonical pathway between risperidone and olanzapine treatments

3.3.1. Acute phase response signaling

The acute phase response proteins in our data suggest a reduction of activation of the acute phase response signaling canonical pathway in the OGR and RGR groups. GWAS studies point to some immune system genes associated with a higher risk for schizophrenia, such as the major histocompatibility complex/human leukocyte antigen MHC/HLA and complement C4 loci [59–62]. MHC/HLA plays a role in coding proteins to bind to antigens on the cell surface, meaning that any alteration in this complex may increase the risk of developing a disease with an immune component [59]. Brain imaging data and mRNA expression in blood of co-twins SCZ and controls showed altered levels of mRNA in C5 associated with cortical thickness in frontal cortex [63]. Consistent evidence suggests that circulating proteins from the immune system, including the acute phase response signaling pathway (e.g., C-reactive protein), are altered in first episode psychosis (FEP) as well as in individuals with a high risk for psychosis [64–67]. Meanwhile, the disruption of these pathways is known to have effects on mood, emotional responses, and cognitive processes via disrupted permeability of the blood-brain barrier, affecting brain cells [68,69]. In addition, a relationship between atypical antipsychotics and an anti-inflammatory effect has been supported by other studies [70–73].

Among the acute phase response signaling proteins, renin angiotensinogen system (RAS) molecules play an important role in cardiovascular homeostasis and cognition improvement. Centrally, angiotensinogen, mostly produced by astrocytes, is secreted and converted to its active form [74]. It was observed that the increase in RAS levels is related to oxidative stress, endothelial dysfunction and inflammation

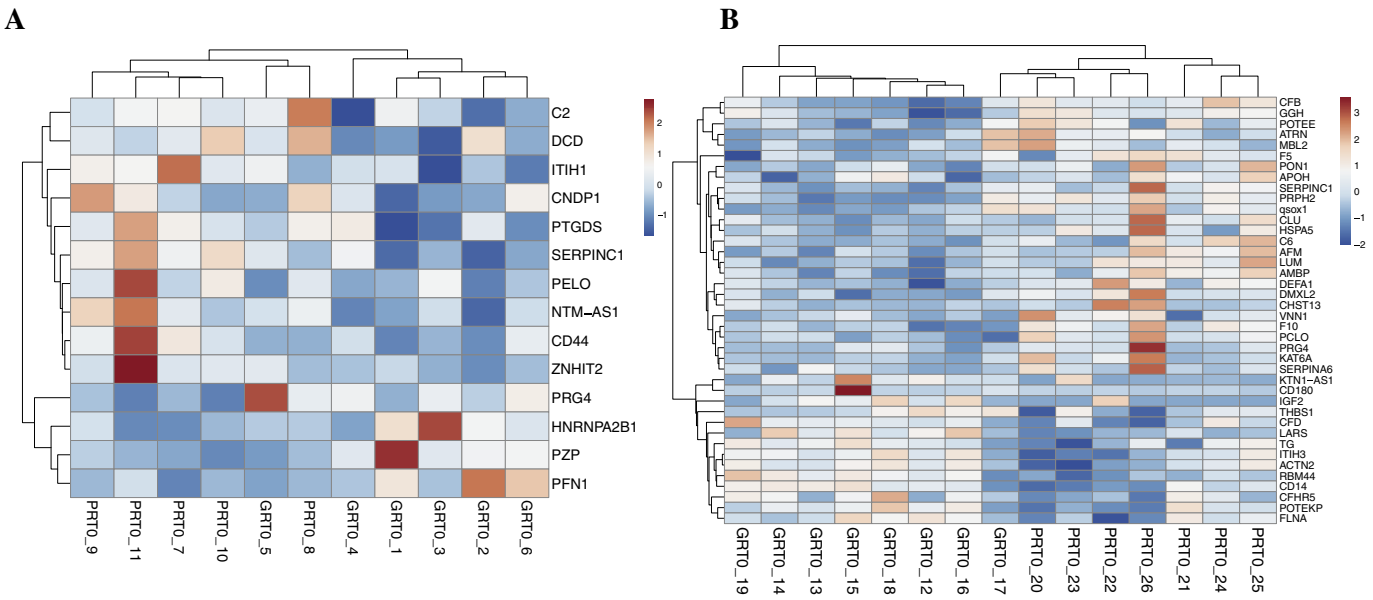

Fig. 3. Hierarchical clustering grouping differentially regulated proteins from good and poor responders to (A) olanzapine and (B) risperidone treatment. The hierarchical cluster trees highlight relationships in the matrix. GR: good responder, PR: poor responder. We applied the default parameters from ClustVis software [42].

[75]. Recent studies suggest that inhibition of RAS may improve cognitive deficits observed in several neurodegenerative disorders [76,77]. In schizophrenia the angiotensinogen risk allele AGT M268T has been found to be altered in whole blood compared to control subjects and is associated with cardiometabolic disorders and cognitive deficits [78]. Also, another study showed a high level of phosphorylated angiotensinogen in blood serum of schizophrenia patients compared with healthy individuals [79]. In our study this protein was decreased in the group of good responders to olanzapine.

Alpha-1 antichimotrypsin is another protein related to acute phase response signaling pathway, that was found to be downregulated in the RGR and OGR groups. This protein is thought to inhibit inflammation by regulation of cathepsin G released at the site of inflammation [80]. Studies of animal models and human postmortem brains and blood of schizophrenia patients have consistently identified alpha-1 antichimotrypsin as being increased compared to control subjects [81–85]. A recent plasma proteome study of patients with schizophrenia showed a decrease in this protein following the use of atypical antipsychotics [26], corroborating our findings.

In line with the above evidence, clinical studies have found that the adjunct administration of an antipsychotic with anti-inflammatory drugs reduces the psychopathologic symptoms of schizophrenia [86–88]. In agreement with the literature, our data suggest a positive effect of olanzapine and risperidone in modulation of this pathway.

3.3.2. Production of nitric oxide (NO) and reactive oxygen species (ROS) in macrophages

The production of NO and ROS in the macrophage canonical pathway was modulated only by OGR and RGR groups in our data, and these were regulated in different directions. This may suggest a distinct mechanism of action for olanzapine, compared with risperidone. NO and ROS are part of crucial processes in the prevention of microorganismal infections and are a source of free radicals, which have been shown to play a role in the pathophysiology of schizophrenia and many other diseases [89,90]. Elevated levels of lipid peroxidation impair the antioxidant defenses in FEP and the levels of this molecule have been found to be reduced after treatment with atypical antipsychotics, improving oxidative damage markers [91–93]. Some antipsychotics, such as olanzapine and risperidone, inhibit the production of pro-inflammatory cytokines, indicating anti-inflammatory properties of these compounds [70]. In addition, clinical trials have shown antioxidant treatment to be effective in improving the symptoms of schizophrenia [94–97].

3.3.3. Complement system

In our study we observed several complement system proteins to be mostly downregulated, in olanzapine and risperidone good responder groups.

The complement proteins are expressed in the periphery and central nervous system, where they are involved in neurogenesis, synaptic pruning and neuronal migration [98], as well as opsonization of foreign molecules, the inflammatory response and lysis of pathogenic microorganisms [99]. Pathological conditions such as neurological diseases may contribute to inflammatory processes through production of cytokines that cross the blood-brain barrier and allow peripheral complement to enter the brain [100]. Also, in schizophrenia the most affected complement protein is C4 and this is suggested to play a role in synaptic pruning [59].

3.3.4. LXR/RXR activation

Our data show a suggestive activation of the LXR/RXR pathway in RGR and decreased activation in OGR and OPR groups. These liver and retinoid receptors play an important role in regulation of lipid metabolism as well as immune and inflammatory responses in macrophages [101]. Several studies and clinical trials have suggested potential targets for the RXR system with positive effects on neurodegeneration and schizophrenia symptoms [102–107]. Further studies should be performed to determine if this pathway is associated with the therapeutic effect of antipsychotics.

3.4. Unique pathways associated with olanzapine treatment

3.4.1. Coagulation system and intrinsic prothrombin activation pathway

Changes in proteins from the coagulation system and intrinsic prothrombin activation canonical pathways were observed in our data, which are predicted to be less activated in OGR, and which might be a potential therapeutic effect of the medication. It is becoming more plausible that coagulation factors are not only involved in the blood

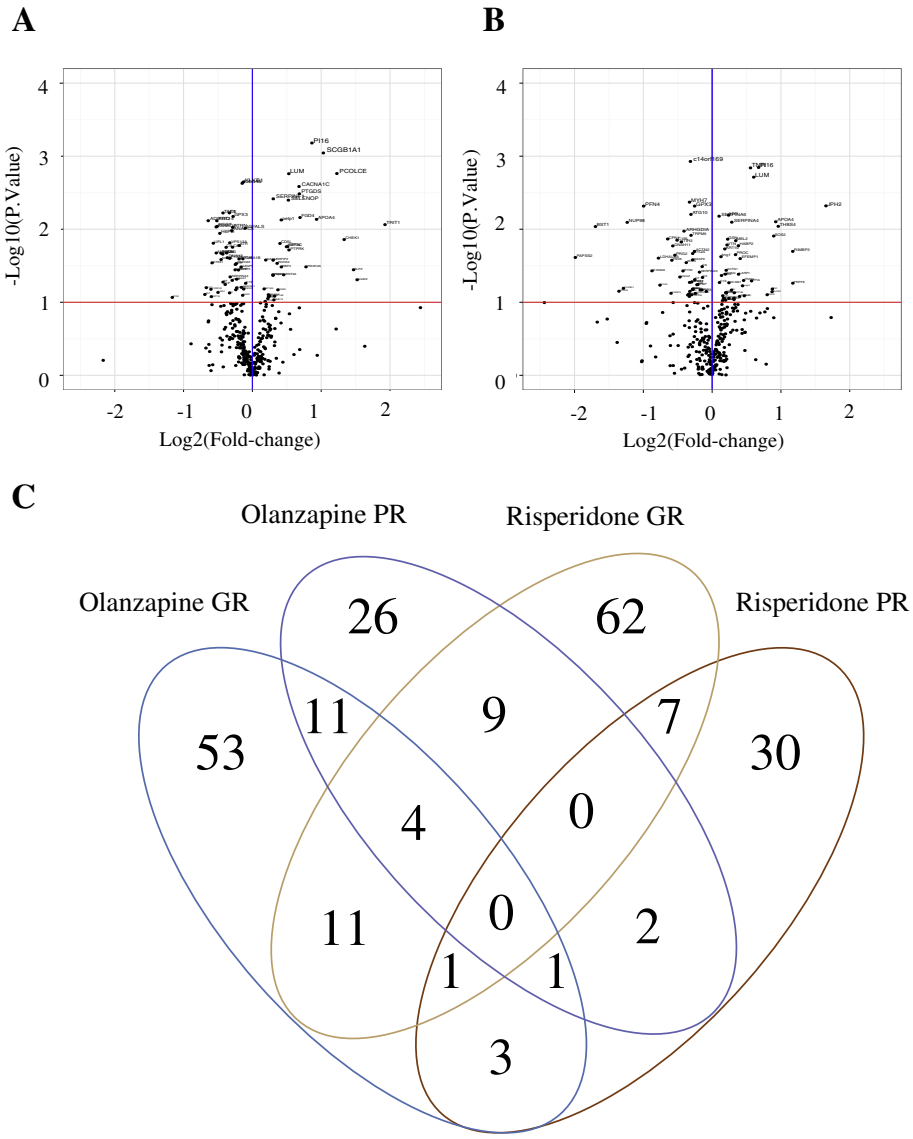

Fig. 4. Data analyses from olanzapine and risperidone T6 vsT0 groups. Volcano plot of quantified proteins from (A) olanzapine and (B) risperidone groups. (C) Venn diagram of differentially regulated proteins in olanzapine and risperidone good and poor responders.

coagulation cascade, but also in processes such as the regulation of myelination and inflammation after brain injury [55,108,109]. Also, there has been an increase in evidence that points to a disruption of coagulation proteins in psychiatric disorders, which have been found to have poor anticoagulation control and prominent phosphorylation differences of multiple proteins in this pathway when compared to controls [107]. This highlights the possible benefits of the chronic use of warfarin treatment for deep-vein thrombosis in schizophrenia patients for reduction of psychotic symptoms [110,111].

3.5. Unique pathways associated with risperidone treatment

3.5.1. Innate immune system and neutrophil degranulation

Changes in proteins from the Innate immune system and neutrophil degranulation pathways were observed in our data.

A cross-sectional study comparing magnetic resonance imaging and blood analysis found an association between reduced brain gray matter and increased levels of neutrophil granulocytes in first episode psychosis compared with heathy controls [112]. The finding that alterations in molecules and cells from the innate immune system, such as high levels neutrophils and monocytes and low levels of eosinophils has been suggested as markers consistent with the immune hypothesis of psychosis in schizophrenia. This was reinforced by the correlation of some of these molecules with higher PANSS-P scores at baseline in a recent study which also found that neutrophil counts were induced by olanzapine treatment [73].

4. Limitations

Of note, the analyses were performed with a number of potential confounding factors, including baseline symptom severity, age, gender, BMI and smoking. In addition, the small sample size, the inherent limitations of the instrumentation and the challenges related to the type of biological sample resulted in a modest number of protein identifications, as observed in similar studies [113–116]. Validation using different sample cohorts could be useful to reinforce the data presented in this exploratory study. Of note, the biological samples used in this study are a subset of a larger cohort, but was selected those which came from drug-naïve or drug-free donors, to exclude possible effects of previous medications at baseline. Also, during the treatment period patients were hospitalized to avoid non informed dropout and analysis bias. Together, these factors increased the reliability of the

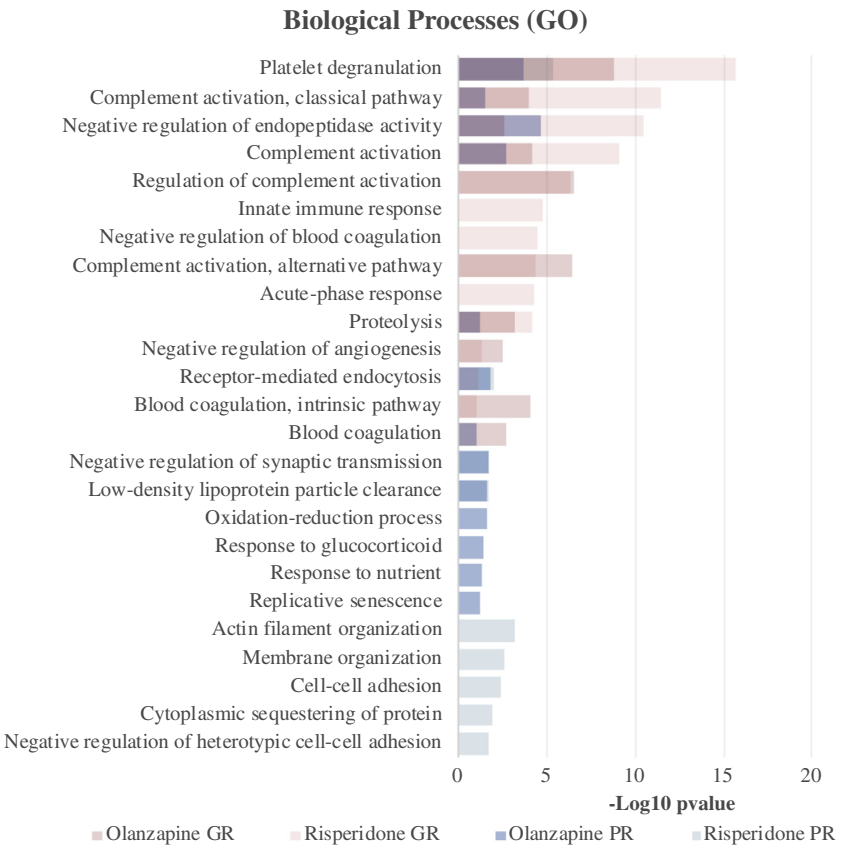

Fig. 5. Biological processes (David direct) considering top 10 biological process of olanzapine and risperidone T6vsT0 groups.

experimental design. With regards to validation, multiplexing approaches such as LC-MS potentially have built in validation through identification of multiple proteins belonging to the same pathway, as we found here. In addition, several of the proteins and pathways that we identified were consistent with those in previously published manuscripts, thus adding a form of cross study validation. Given these points, we intended this as an exploratory study as a basis for new investigations aimed at identifying antipsychotic response biomarkers in

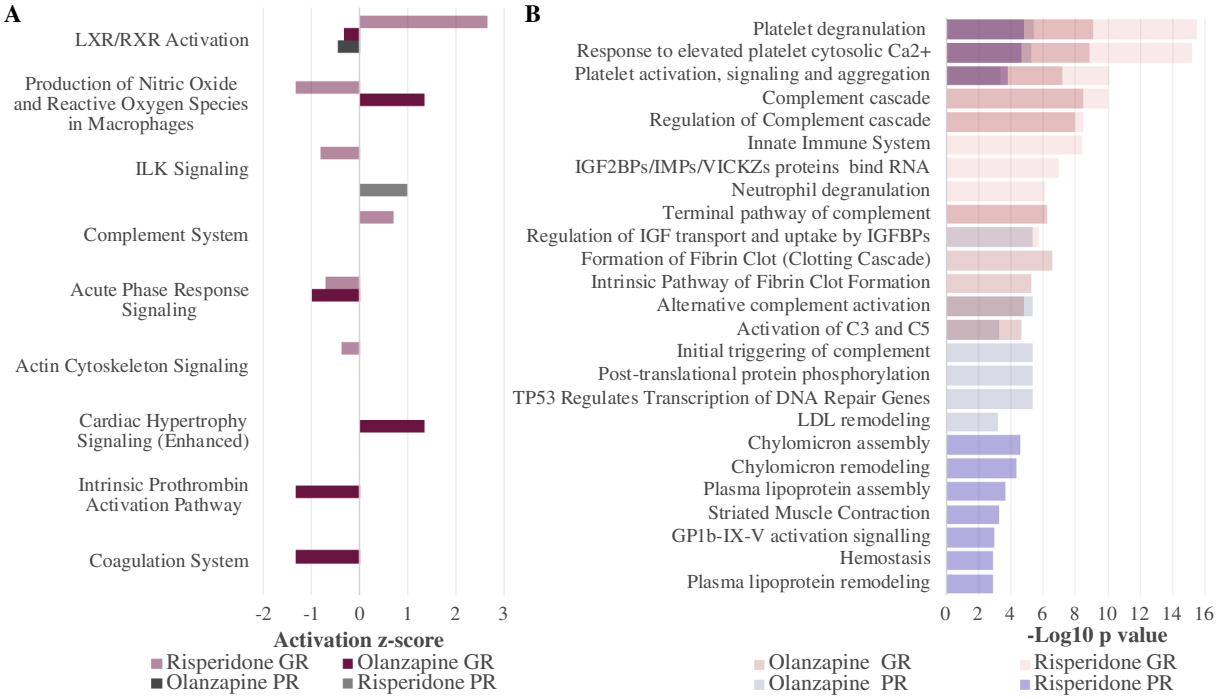

Fig. 6. Pathway analyses of proteins differentially abundant between T6 versus T0, from GR and PR risperidone and olanzapine groups. Canonical pathway analyses from (A) IPA and (B) Reactome.

Table 3
Pathways affected by olanzapine and risperidone good responders in T6 vs T0 analysis.

Group	Accession number	Gene name	Description name	Regulation	Pathways
OGR	P21549	AGT	Serine–pyruvate aminotransferase	↓	acute phase response signaling; activation of LXR/RXR
OGR	P06727	APOA4	Apolipoprotein A-IV	↑	production of nitric oxide and reactive oxygen species in macrophages;activation of LXR/RXR
OGR	Q13790	APOF	Apolipoprotein F	↓	production of nitric oxide and reactive oxygen species in macrophages; activation of LXR/RXR
OGR	P02749	APOH	Beta-2-glycoprotein 1	↓	acute phase response signaling; activation of LXR/RXR
OGR	P09871	C1S	Complement C1s subcomponent	↓	acute phase response signaling; complement system
OGR	P06681	C2	Complement C2	↓	acute phase response signaling; complement system
OGR	P13671	C6	Complement component C6	↓	complement system
OGR	P07357	C8A	Complement component C8 alpha chain	↓	complement system
OGR	P07360	C8G	Complement component C8 gamma chain	↓	complement system
OGR	P02748	C9	Complement component C9	↓	acute phase response signaling; complement system; activation of LXR/RXR
OGR	P00746	CFD	Complement factor D	↑	complement system
OGR	P36980	CFHR2	Complement factor H-related protein 2	↓	complement system
OGR	P27918	CFP	Properdin	↓	complement system
OGR	P00450	CP	Ceruloplasmin	↓	acute phase response signaling
OGR	P00488	F13A1	Coagulation factor XIII A chain	↓	prothrombin activation; coagulation system/clotting cascade
OGR	P00734	F2	Prothrombin	↓	prothrombin activation; coagulation system/clotting cascade; acute phase response signaling; complement system
OGR	P12259	F5	Coagulation factor V	↓	prothrombin activation; coagulation system/clotting cascade
OGR	P07359	GP1BA	Platelet glycoprotein Ib alpha chain	↑	coagulation system/clotting cascade
OGR	P03952	KLKB1	Plasma kallikrein	↓	prothrombin activation; coagulation system/clotting cascade; acute phase response signaling; complement system
OGR	O00443	PIK3C2A	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha	↑	production of nitric oxide and reactive oxygen species in macrophages
OGR	P27169	PON1	Serum paraoxonase/arylesterase 1	↑	production of nitric oxide and reactive oxygen species in macrophages; activation of LXR/RXR
OGR	P02753	RBP4	Retinol-binding protein 4	↑	acute phase response signaling; production of nitric oxide and reactive oxygen species in macrophages; activation of LXR/RXR
OGR	P01011	SERPINA3	Alpha-1-antichymotrypsin	↓	acute phase response signaling
OGR	P01008	SERPINC1	Antithrombin-III	↓	prothrombin activation; coagulation system/clotting cascade
OGR	P36955	SERPINF1	Pigment epithelium-derived factor	↑	acute phase response signaling; activation of LXR/RXR
OGR	P02787	TF	Serotransferrin	↓	acute phase response signaling; activation of LXR/RXR
RGR	P04217	A1BG	Alpha-1B-glycoprotein	↑	innate immune system; neutrophil degranulation; complement system; LXR/RXR activation
RGR	P02765	AHSG	Alpha-2-HS-glycoprotein	↑	acute phase response signaling; innate immune system; neutrophil degranulation; complement system; LXR/RXR activation
RGR	P02652	APOA2	Apolipoprotein A-II	↓	acute phase response signaling; production of nitric oxide and reactive oxygen species in macrophages; LXR/RXR activation
RGR	P06727	APOA4	Apolipoprotein A-IV	↑	production of nitric oxide and reactive oxygen species in macrophages; LXR/RXR activation
RGR	Q13790	APOF	Apolipoprotein F	↓	production of nitric oxide and reactive oxygen species in macrophages; LXR/RXR activation
RGR	P02749	APOH	Beta-2-glycoprotein 1	↑	LXR/RXR activation
RGR	Q10588	BST1	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2	↓	innate immune system; neutrophil degranulation; complement system; LXR/RXR activation; complement system
RGR	P02745	C1QA	Complement C1q subcomponent subunit A	↓	innate immune system; complement system
RGR	P02746	C1QB	Complement C1q subcomponent subunit B	↑	innate immune system; complement system
RGR	P00736	C1R	Complement C1r subcomponent	↑	acute phase response signaling; innate immune system; complement system
RGR	P09871	C1S	Complement C1s subcomponent	↑	acute phase response signaling; innate immune system; complement system
RGR	P01024	C3	Complement C3	↑	acute phase response signaling; innate immune system; neutrophil degranulation; complement system; LXR/RXR activation;
RGR	P01031	C5	Complement C5	↓	acute phase response signaling; innate immune system; complement system
RGR	P13671	C6	Complement component C6	↓	innate immune system; complement system
RGR	P10643	C7	omplement component C7	↓	innate immune system; complement system
RGR	P07360	C8G	Complement component C8 gamma chain	↓	innate immune system; complement system
RGR	P08571	CD14	Monocyte differentiation antigen CD14	↓	innate immune system; neutrophil degranulation; complement system; LXR/RXR activation
RGR	P16070	CD44	CD44 antigen	↓	innate immune system; neutrophil degranulation; complement system
RGR	P00450	CP	Ceruloplasmin	↓	acute phase response signaling
RGR	P15169	CPN1	Carboxypeptidase N catalytic chain	↓	innate immune system; neutrophil degranulation; complement system
RGR	P54108	CRISP3	Cysteine-rich secretory protein 3	↓	innate immune system; neutrophil degranulation; complement system
RGR	P02751	FN1	Fibronectin	↑	acute phase response signaling
RGR	P06396	GSN	Gelsolin	↑	innate immune system; neutrophil degranulation; complement system
RGR	P19823	ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	↑	acute phase response signaling
RGR	Q06033	ITIH3	Inter-alpha-trypsin inhibitor heavy chain H3	↓	acute phase response signaling
RGR	P01042	KNG1	Kininogen-1	↑	LXR/RXR activation
RGR	P13473	LAMP2	Lysosome-associated membrane glycoprotein 2	↓	neutrophil degranulation; complement system
RGR	P02750	LRG1	Leucine-rich alpha-2-glycoprotein	↓	innate immune system; neutrophil degranulation; complement system
RGR	O00187	MASP2	Mannan-binding lectin serine protease 2	↓	innate immune system; complement system

(*continued on next page*)

Table 3 (*continued*)

Group	Accession number	Gene name	Description name	Regulation	Pathways
RGR	P11226	MBL2	Mannose-binding protein C	↑	acute phase response signaling; innate immune system; complement system
RGR	Q9UKX2	MYH2	Myosin-2	↓	innate immune system; complement system
RGR	P19838	NFKB1	Nuclear factor NF-kappa-B p105 subunit	↓	acute phase response signaling; production of nitric oxide and reactive oxygen species in macrophages; innate immune system; neutrophil degranulation; complement system; LXR/RXR activation
RGR	P00747	PLG	Plasminogen	↓	acute phase response signaling
RGR	P02775	PPBP	Platelet basic protein	↓	innate immune system; neutrophil degranulation; complement system
RGR	P62937	PPIA	Peptidyl-prolyl cis-trans isomerase A	↑	innate immune system; neutrophil degranulation; complement system
RGR	P13727	PRG2	Bone marrow proteoglycan	↓	innate immune system; neutrophil degranulation; complement system
RGR	P05109	S100A8	Protein S100-A8	↓	production of nitric oxide and reactive oxygen species in macrophages; innate immune system; neutrophil degranulation; complement system; LXR/RXR activation
RGR	P0DJI8	SAA1	Serum amyloid A-1 protein	↑	acute phase response signaling; innate immune system; LXR/RXR activation
RGR	P01011	SERPINA3	Alpha-1-antichymotrypsin	↓	acute phase response signaling; innate immune system; neutrophil degranulation; complement system
RGR	P36955	SERPINF1	Pigment epithelium-derived factor	↑	acute phase response signaling; LXR/RXR activation
RGR	Q07890	SOS2	Son of sevenless homolog 2	↑	acute phase response signaling
RGR	P04004	VTN	Vitronectin	↑	innate immune system; LXR/RXR activation

OGR = olanzapine good responder; RGR = risperidone good responder; ↑ = up-regulation; ↓ = Down-regulation.

longitudinal analyses of different cohorts.

5. Conclusion

This exploratory, longitudinal profiling study of blood plasma samples from patients with paranoid schizophrenia provided evidence of significant changes in protein levels mostly related to the immune and inflammatory systems in response to antipsychotic treatment. The study also led to identification of a panel of differentially abundant proteins that may be associated with a positive outcome for olanzapine and risperidone treatments in patients with schizophrenia before treatment. By analyzing the plasma from peripheral blood using 2D-UPLC-HDMSE of those patients who exhibited a good or poor outcome to those atypical antipsychotics, prior to medication, we were able to find potential signatures to predict this efficiency. The altered levels of some proteins are known to be involved in the pathophysiology of schizophrenia. This is an initial study and if confirmed in a larger cohort, the panel of proteins might become a signature used to predict a positive outcome in early schizophrenia diagnosis. This could help to prevent progression of the disorder by improving treatment options, thereby improving the quality of life of patients and reducing hospital costs.

Despite the study limitations discussed above, this is one of the first studies to identify differential enrichment of these pathways in good responders to the olanzapine and risperidone treatments, suggesting a role in the therapeutic effects of these medications. These proteins were mainly associated with acute phase response signaling, the coagulation system, intrinsic prothrombin activation, production of NO and ROS in macrophages, and LXR/RXR activation pathways. Considering this as novel information on the specific proteins involved, and with partial validation of the findings achieved through pathway enrichment analysis, these studies could lead to identification of new targets and co-treatments might be considered to treat the disease. Our findings reinforce the concept that the immune component, which has been already suggested to be associated with the pathophysiology of SCZ, can be modulated by atypical antipsychotics. This continues to be a target for the study of peripheral signaling modulated by antipsychotics in order to better understand their biological mechanisms and also to develop a potential panel of surrogate protein markers which may be associated with treatment response.

Data availability

The mass spectrometry proteomic data have been deposited into the ProteomeXchange Consortium via the PRIDE [117] partner repository with the dataset identifier PXD015049 and PXD015213".

Author contributions

DM-d-S conceived, designed and supervised the study. SG-R executed the experiments, analyzed obtained data and wrote the manuscript. BC provided support on statistical analysis. PCG reviewed the manuscript and helped in data interpretation. JS provided blood plasma samples and the clinical information from SCZ patients and helped in data interpretation. All authors contributed to and approved the final manuscript.

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Declaration of Competing Interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.103813.

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